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(54) Title: KUNITZ TYPE PROTEASE INHIBITORS			
(57) Abstract Analogues of the Kunitz Protease Inhibitor (KPI) domain of amyloid precursor protein bind to and inhibit activity of serine proteases, including kallikrein, plasmin and coagulation factors such as factors VIIa, IXa, Xa, XIa and XIIa. Pharmaceutical compositions containing the KPI analogues, along with methods for using such compositions, are useful for ameliorating and treating clinical conditions associated with increased serine protease activity, such as blood loss related to cardiopulmonary bypass surgery. Nucleic acid sequences encoding these analogues and systems for expression of the peptides of the invention are provided.			

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KUNITZ TYPE PROTEASE INHIBITORS

Background of the Invention

The plasma, or serine, proteases of the blood contact system are known to be activated by interaction with negatively charged surfaces. For example, tissue injury during surgery exposes the vascular basement membrane, causing interaction of the blood with collagen, which is negatively charged at physiological pH. This induces a cascade of proteolytic events, leading to production of plasmin, a fibrinolytic protease, and consequent blood loss.

Perioperative blood loss of this type can be particularly severe during cardiopulmonary bypass (CPB) surgery, in which the patient's blood flow is diverted to an artificial heart-lung machine. CPB is an essential component of a number of life-saving surgical procedures. For example, in the United States, it is estimated that 300,000 patients every year undergo coronary artery bypass grafts involving the use of CPB.

Although necessary and generally safe, CPB is associated with a significant rate of morbidity, some of which may be attributed to a "whole body inflammatory response" caused by activation of plasma protease systems and blood cells through interactions with the artificial surfaces of the heart-lung machine (Butler et al., *Ann. Thorac. Surg.* 55:552 (1993); Edmunds et al., *J. Card. Surg.* 8:404 (1993)). For example, during extracorporeal circulation, exposure of blood to negatively charged surfaces of the artificial bypass circuit, e.g., plastic surfaces in the heart-lung machine, results in direct activation of plasma factor XII.

Factor XII is a single-chain 80 kDa protein that circulates in plasma as an inactive zymogen. Contact with negatively charged nonendothelial surfaces, like those of the bypass circuit, causes surface-bound factor XII to be autoactivated to the active serine protease factor XIIa. See C lman, *Agents Actions Suppl.* 42:125

(1993). Surface-activated factor XIIa then processes prekallikrein (PK) to active kallikrein, which in turn cleaves more XIIa from XII in a reciprocal activation reaction that results in a rapid amplification of the contact pathway. Factor XIIa can also activate the first component of complement C1, leading to production of the anaphylatoxin C5a through the classical complement pathway.

The CPB-induced inflammatory response includes changes in capillary permeability and interstitial fluid accumulation. Cleavage of high molecular weight kininogen (HK) by activated kallikrein generates the potent vasodilator bradykinin, which is thought to be responsible for increasing vascular permeability, resulting in edema, especially in the lung. The lung is particularly susceptible to damage associated with CPB, with some patients exhibiting what has been called "pump lung syndrome" following bypass, a condition indistinguishable from adult respiratory distress. See Johnson et al., *J. Thorac. Cardiovasc. Surg.* 107:1193 (1994).

Post-CPB pulmonary injury includes tissue damage thought to be mediated by neutrophil sequestration and activation in the microvasculature of the lung. (Butler et al., *supra*; Johnson, et al., *supra*). Activated factor XII can itself stimulate neutrophil aggregation. Factor XIIa-generated kallikrein, and complement protein C5a generated by Factor XIIa activation of the complement cascade, both induce neutrophil chemotaxis, aggregation and degranulation. See Edmunds et al., *supra* (1993). Activated neutrophils may damage tissue through release of oxygen-derived free-radicals, proteolytic enzymes such as elastase, and metabolites of arachidonic acid. Release of neutrophil products in the lung can cause changes in vascular tone, endothelial injury and loss of vascular integrity.

Intrinsic inhibition of the contact system occurs through inhibition of activated XIIa by C1-inhibitor (C1-INH). See C lman, *supra*. During CPB, this natural

inhibitory mechanism is overwhelmed by massive activation of plasma proteases and consumption of inhibitors. A potential therapeutic strategy for reducing post-bypass pulmonary injury mediated by neutrophil activation would, therefore, be to block the formation and activity of the neutrophil agonists kallikrein, factor XIIa, and C5a by inhibition of proteolytic activation of the contact system.

Protease inhibitor therapy which partially attenuates the contact system is currently employed clinically in CPB. Aprotinin, also known as basic pancreatic protease inhibitor (BPI), is a small, basic, 58 amino acid polypeptide isolated from bovine lung. It is a broad spectrum serine protease inhibitor of the Kunitz type, and was first used during bypass in an attempt to reduce the inflammatory response to CPB. See Butler et al., *supra*. Aprotinin treatment results in a significant reduction in blood loss following bypass, but does not appear to significantly reduce neutrophil activation. Additionally, since aprotinin is of bovine origin, there is concern that repeated administration to patients could lead to the development of an immune response to aprotinin in the patients, precluding its further use.

The proteases inhibited by aprotinin during CPB appear to include plasma kallikrein and plasmin. (See, e.g., Scott, et al., *Blood* 69:1431 (1987)). Aprotinin is an inhibitor of plasmin (K_i of 0.23nM), and the observed reduction in blood loss may be due to inhibition of fibrinolysis through the blocking of plasmin action. Although aprotinin inhibits plasma kallikrein, (K_i of 20nM), it does not inhibit activated factor XII, and consequently only partially blocks the contact system during CPB.

Another attractive protease target for use of protease inhibitors, such as those of the present invention, is factor XIIa, situated at the very first step of contact activation. By inhibiting the proteolytic activity of factor XIIa, kallikrein production would be prevented, blocking amplification of

the contact system, neutrophil activation and bradykinin release. Inhibition of XIIa would also prevent complement activation and production of C5a. More complete inhibition of the contact system during CPB could, therefore, be achieved through the use of a better XIIa inhibitor.

Protein inhibitors of factor XIIa are known. For example, active site mutants of α_1 -antitrypsin that inhibit factor XIIa have been shown to inhibit contact activation in human plasma. See Patston et al., *J. Biol. Chem.* 265:10786 (1990). The large size and complexity (greater than 400 amino acid residues) of these proteins present a significant challenge for recombinant protein production, since large doses will almost certainly be required during CPB. For example, although it is a potent inhibitor of both kallikrein and plasmin, nearly 1 gram of aprotinin must be infused into a patient to inhibit the massive activation of the kallikrein-kinin and fibrinolytic systems during CPB.

The use of smaller, more potent XIIa inhibitors such as the corn and pumpkin trypsin inhibitors (Wen, et al., *Protein Exp. & Purif.* 4:215 (1993); Pedersen, et al., *J. Mol. Biol.* 236:385 (1994)) could be more cost-effective than the large α_1 -antitrypsins, but the infusion of high doses of these non-mammalian inhibitors could result in immunologic reactions in patients undergoing repeat bypass operations. The ideal protein XIIa inhibitor is, therefore, preferably, small, potent, and of human sequence origin.

One candidate for an inhibitor of human origin is found in circulating isoforms of the human amyloid β -protein precursor (APPI), also known as protease nexin-2. APPI contains a Kunitz serine protease inhibitor domain known as KPI (Kunitz Protease Inhibitor). See Ponte et al., *Nature*, 331:525 (1988); Tanzi et al., *Nature* 331:528 (1988); Johnstone et al., *Biochem. Biophys. Res. Commun.* 163:1248 (1989); Oltersdorf et al., *Nature* 341:144 (1989). Human KPI shares about 45% amino acid sequence identity with aprotinin. The isolated KPI domain has

been prepared by recombinant expression in a variety of systems, and has been shown to be an active serine protease inhibitor. See, for example, Sinha, et al., *J. Biol. Chem.* 265:8983 (1990). The measured in vitro K_i of KPI against plasma kallikrein is 45nM, compared to 20nM for aprotinin.

Aprotinin, KPI, and other Kunitz-type serine protease inhibitors have been engineered by site-directed mutagenesis to improve inhibitory activity or specificity. Thus, substitution of Lys¹⁵ of aprotinin with arginine resulted in an inhibitor with a K_i of 0.32nM toward plasma kallikrein, a 100-fold improvement over natural aprotinin. See PCT application No. 89/10374. See also Norris et al., *Biol. Chem. Hoppe Seyler* 371:3742 (1990). Alternatively, substitution of position 15 of aprotinin with valine or substitution of position 13 of KPI with valine resulted in elastase inhibitors with K_s in the 100 pM range, although neither native aprotinin nor native KPI significantly inhibits elastase. See Wenzel et al., in: *Chemistry of Peptides and Proteins*, Vol. 3, (Walter de Gruyter, Berlin, New York, 1986); Sinha et al., *supra*. Methods for substituting residues 13, 15, 37, and 50 of KPI are shown in general terms in European Patent Application No. 0 393 431, but no specific sequences are disclosed, and no protease inhibition data are given.

Phage display methods have been recently used for preparing and screening derivatives of Kunitz-type protease inhibitors. See PCT Application No. 92/15605, which describes specific sequences for 34 derivatives of aprotinin, some of which were reportedly active as elastase and cathepsin inhibitors. The amino acid substitutions in the derivatives were distributed throughout almost all positions of the aprotinin molecule.

Phage display methods have also been used to generate KPI variants that inhibit factor VIIa and kallikrein. See Dennis et al., *J. Biol. Chem.* 269:22129 and 269:22137 (1994). The residues that could be varied in the phage

display selection process were limited to positions 9-11, 13-17, 32, 36 and 37, and several of those residues were also held constant for each selection experiment. One of those variants was said to have a K_i of 1.2nM for kallikrein, and had substitutions at positions 9 (Thr→Pro), 13 (Arg→Lys), 15 (Met→Leu), and 37 (Gly→Tyr). None of the inhibitors was tested for the ability to inhibit factor XIIa.

It is apparent, therefore, that new protease inhibitors that can bind to and inhibit the activity of serine proteases are greatly to be desired. In particular it is highly desirable to prepare peptides, based on human peptide sequences, that can inhibit selected serine proteases such as kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. It is also highly desirable to prepare novel protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example by reducing pulmonary damage or blood loss during CPB.

Summary of the Invention

The present invention relates to peptides that can bind to and preferably exhibit inhibition of the activity of serine proteases. Those peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. Particularly, the novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest in comparison to known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly

those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

5 In achieving the inhibition of serine protease activity, the invention provides protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example, by reducing pulmonary damage or
10 blood loss during CPB.

The present invention relates to protease inhibitors comprising the following amino acid sequences:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-X³-Cys-Arg-
Ala-X⁴-X⁵-X⁶-X⁷-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-
15 Lys-Cys-Ala-Pro-Phe-X⁸-Tyr-Gly-Gly-Cys-X⁹-X¹⁰-X¹¹-
X¹²-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-
Val-Cys-Gly-Ser-Ala-Ile,

wherein: X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X² is selected from Thr, Val, Ile and Ser; X³ is
20 selected from Pro and Ala; X⁴ is selected from Arg, Ala, Leu, Gly, or Met; X⁵ is selected from Ile, His, Leu, Lys, Ala, or Phe; X⁶ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁷ is selected from Arg, His, or Ala; X⁸ is selected from Phe, Val, Leu, or Gly;
25 X⁹ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X¹⁰ is selected from Ala, Arg, or Gly; X¹¹ is selected from Lys, Ala, or Asn; and X¹² is selected from Ser, Ala, or Arg.

The invention relates more specifically to protease
30 inhibitors comprising the following amino acid sequences:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-X³-Cys-Arg-
Ala-X⁴-X⁵-X⁶-X⁷-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-
Lys-Cys-Ala-Pro-Phe-X⁸-Tyr-Gly-Gly-Cys-X⁹-X¹⁰-X¹¹-
X¹²-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-
35 Val-Cys-Gly-Ser-Ala-Ile,

wherein X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X² is selected from Thr, Val, Ile and Ser; X³ is selected from Pro and Ala; X⁴ is selected from Arg, Ala, Leu, Gly, or Met; X⁵ is selected from Ile, His, Leu, Lys,

Ala, or Phe; X⁴ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁷ is selected from Arg, His, or Ala; X⁸ is selected from Phe, Val, Leu, or Gly; X⁹ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X¹⁰ is selected from Ala, Arg, or Gly; X¹¹ is selected from Lys, Ala, or Asn; X¹² is selected from Ser, Ala, or Arg; provided that when X⁴ is Arg, X⁵ is Ile; when X⁵ is Arg, X⁴ is Ala or Leu; when X⁵ is Tyr, X⁴ is Ala or X³ is His; and either X³ is not Ile; or X⁴ is not Ser; or X⁵ is not Leu, Phe, Met, Tyr, or Asn; or X¹⁰ is not Gly; or X¹¹ is not Asn; or X¹² is not Arg.

Another aspect of this invention provides protease inhibitors wherein at least two amino acid residues selected from the group consisting of X⁴, X⁵, X⁶, and X⁷ defined above differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides protease inhibitors wherein X¹ is Asp or Glu, X² is Thr, X³ is Pro, and X¹² is Ser. Yet another aspect of this invention provides protease inhibitors wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, and X¹¹ is Asn. Another aspect of this invention provides protease inhibitors wherein X¹ is Asp, X² is Thr, X³ is Pro, X⁴ is Arg, X⁵ is Ile, X⁶ is Ile, X⁷ is Arg, X⁸ is Val, X⁹ is Arg, X¹⁰ is Ala, and X¹¹ is Lys. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Ala. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Ala, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is

Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Arg, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Val, Leu, or Gly, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Ala, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Pro, X⁴ is Ala or Leu, X⁵ is Ile, X⁶ is Tyr, X⁷ His, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

Yet another aspect of this invention provides protease inhibitors wherein X² is Thr, and X⁴ is Ala. Another aspect of this invention provides protease inhibitors wherein X² is Thr, and X⁴ is Leu. Another aspect of this invention provides protease inhibitors wherein X² is Val, and X⁴ is Ala. Another aspect of this invention provides protease inhibitors wherein X² is Ser, and X⁴ is Ala. Another aspect of this invention provides protease inhibitors wherein X² is Val, and X⁴ is Leu. Another aspect of this invention provides protease inhibitors wherein X² is Ser, and X⁴ is Leu.

Yet another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Tyr, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg. Another aspect of this invention provides protease inhibitors wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Leu, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

The present invention also relates to protease inhibitors comprising the following amino acid sequences:

5 X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-
Pro-Cys-X¹-Ala-X²-X³-X⁴-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-
Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-
Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-
Cys-Gly-Ser-Ala-Ile,

10 wherein: X¹ is selected from Glu-Val-Val-Arg-Glu- and
Asp-Val-Val-Arg-Glu-; X² is selected from Arg and Lys; X³
is selected from Met, Arg, Ala, Leu, Ser, Val; X⁴ is
selected from Ile and Ala; X⁵ is selected from Ser, Ile,
Ala, Pro, Phe, Tyr, and Trp; and X⁶ is selected from Arg,
Ala, His, Gln, and Thr; provided that: when X¹ is Arg, X²
15 is Leu, and X⁴ is Ile, X⁵ cannot be Ser; and also provided
that either X³ is not Met; or X⁴ is not Ile; or X⁵ is not
Ser; or X⁶ is not Arg. Another aspect of this invention
provides protease inhibitors wherein X¹ is Arg or Met,
and X² is Ser or Ile. Yet another aspect of this
20 invention provides protease inhibitors wherein X³ is
selected from Phe, Tyr and Trp. Another aspect of this
invention provides protease inhibitors wherein X⁴ is Ala
or Leu.

A further aspect of this invention provides an
25 isolated DNA molecule comprising a DNA sequence encoding
a protease inhibitor of the invention. Another aspect of
this invention provides an isolated DNA molecule
comprising a DNA sequence encoding the protease inhibitor
that further comprises an isolated DNA molecule operably
30 linked to a regulatory sequence that controls expression
of the coding sequence of the protease inhibitor in a
host cell. Another aspect of this invention provides an
isolated DNA molecule comprising a DNA sequence encoding
the protease inhibitor operably linked to a regulatory
35 sequence that controls expression of the coding sequence
of the protease inhibitor in a host cell that further
comprises a DNA sequence encoding a secretory signal
peptide. That secretory signal peptide may preferably
comprise the signal sequence of yeast alpha-mating

factor. Another aspect of this invention provides a host cell transformed with any of the DNA molecules defined above. Such a host cell may preferably comprise *E. coli* or a yeast cell. When such a host cell is a yeast cell, the yeast cell may be selected from *Saccharomyces cerevisiae* and *Pichia pastoris*.

Another aspect of this invention provides a method for producing a protease inhibitor of the present invention, comprising the steps of culturing a host cell as defined above and isolating and purifying said protease inhibitor.

A further aspect of this invention provides a pharmaceutical composition, comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

An additional aspect of this invention provides a method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle. That method of treatment may preferably be used to treat the clinical condition of blood loss during surgery.

Yet another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

Another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle, wherein said serine proteases

are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; 5 proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

A further aspect of this invention relates to protease inhibitors comprising the following amino acid sequences: 10

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X²-X³-X⁴-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X⁵-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-15 Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X² is selected from Ala, Leu, Gly, or Met; X³ is selected from Ile, His, Leu, Lys, Ala, or Phe; X⁴ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁵ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; provided that when X³ is Arg, X² is Ala or Leu; when X⁴ is Tyr, X² is Ala or X³ is His; and either X³ is not Ile; or X⁴ is not Ser; or X⁵ is not Leu, Phe, Met, Tyr, or Asn. Another aspect of this invention provides a protease inhibitor as defined above wherein X¹ 20 is Glu, X² is Met, X³ is Ile, X⁴ is Ile, and X⁵ is Gly.

The invention also relates more specifically to protease inhibitors comprising the following amino acid sequences:

30 Glu-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X¹-X²-X³-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X⁴-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

35 wherein X¹ is selected from Ala, Leu, Gly, or Met; X² is selected from Ile, His, Leu, Lys, Ala, or Phe; X³ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X⁴ is selected from Gly, Arg, Leu, Met, or Tyr; provided that when X¹ is Ala, X² is Ile, His, r

Leu; when X¹ is Leu, X² is Ile or His; when X¹ is Leu and X² is Ile, X³ is not Ser; when X¹ is Gly, X² is Ile; when X⁴ is Arg, X¹ is Ala or Leu; when X⁴ is Tyr, X¹ is Ala or X² is His; and either X¹ is not Met, or X² is not Ile, or X³ is not Ser, or X⁴ is not Gly.

A further aspect of this invention provides a protease inhibitor as defined above wherein X¹ is Met, X² is Ser, and X⁴ is Gly. Another aspect of this invention provides a protease inhibitor wherein X² is selected from His, Ala, Phe, Lys, and Leu. Another aspect of this invention provides a protease inhibitor wherein X¹ is His. Another aspect of this invention provides a protease inhibitor wherein X¹ is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Phe. Another aspect of this invention provides a protease inhibitor wherein X² is Lys. Another aspect of this invention provides a protease inhibitor wherein X² is Leu. Another aspect of this invention provides a protease inhibitor wherein X¹ is Met, X² is Ile, and X⁴ is Gly.

Yet another aspect of this invention provides a protease inhibitor wherein X² is Ile. Another aspect of this invention provides a protease inhibitor wherein X² is Pro. Another aspect of this invention provides a protease inhibitor wherein X² is Phe. Another aspect of this invention provides a protease inhibitor wherein X² is Tyr. Another aspect of this invention provides a protease inhibitor wherein X² is Trp. Another aspect of this invention provides a protease inhibitor wherein X² is Asn. Another aspect of this invention provides a protease inhibitor wherein X² is Leu.

An additional aspect of this invention provides a protease inhibitor wherein X² is Lys. Another aspect of this invention provides a protease inhibitor wherein X² is His. Another aspect of this invention provides a protease inhibitor wherein X² is Glu. Another aspect of this invention provides a protease inhibitor wherein X¹ is Ala. Another aspect of this invention provides a

protease inhibitor wherein X^1 is Ile. Another aspect of this invention provides a protease inhibitor wherein X^1 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Trp, and X^4 is Gly.

Yet another other aspect of this invention provides a protease inhibitor wherein X^1 is Ser or Phe, and X^4 is Arg or Tyr. Another aspect of this invention provides a protease inhibitor wherein X^1 is His or Leu, X^2 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^1 is His, X^2 is Asn or Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Ile, X^2 is Pro, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Gly, X^2 is Ile, X^3 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Met, X^2 is His, X^3 is Ser, and X^4 is Tyr.

Additionally, another aspect of this invention relates to protease inhibitors comprising the following amino acid sequences:

X^1 -Val-Cys-Ser-Glu-Gln-Ala-Glu- X^2 -Gly-Pro-Cys-Arg-Ala- X^3 - X^4 - X^5 -Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys- X^6 -Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Arg, Ala, Leu, Gly, or Met; X^4 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^5 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^6 is selected from Arg, His, or Ala; and X^7 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr.

Another aspect of this invention provides a protease inhibitor as defined above wherein at least two amino acid residues selected from the group consisting of X^1 ,

X⁴, X⁵, and X⁶ differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Ala or Leu, X⁴ is Ile, X⁵ is Tyr, X⁶ is His and X⁷ is Gly. Another aspect of this invention provides a protease inhibitor wherein X² is Thr, and X³ is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Thr, and X³ is Leu. Another aspect of this invention provides a protease inhibitor wherein X² is Val, and X³ is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Ser, and X³ is Ala. Another aspect of this invention provides a protease inhibitor wherein X² is Val, and X³ is Leu. Another aspect of this invention provides a protease inhibitor wherein X² is Ser, and X³ is Leu. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Gly. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Tyr. Another aspect of this invention provides a protease inhibitor wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Leu.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

35

Brief Description of the Drawings

Figure 1 shows the strategy for the construction of plasmid pTW10:KPI.

Figure 2 shows the sequence of the synthetic gene for KPI (1-57) fused to the bacterial *phoA* secretory signal sequence.

5 Figure 3 shows the strategy for construction of plasmid pKPI-61.

Figure 4 shows the 192 bp *XbaI-HindIII* synthetic gene fragment encoding KPI (1-57) and four amino acids from yeast alpha-mating factor.

10 Figure 5 shows the synthetic 201 bp *XbaI-HindIII* fragment encoding KPI(-4-57) in pKPI-61.

Figure 6 shows the strategy for the construction of plasmid pTW113.

Figure 7 shows plasmid pTW113, encoding the 445 bp synthetic gene for yeast alpha-factor-KPI(-4-57) fusion.

15 Figure 8 shows the amino acid sequence for KPI (-4-57).

Figure 9 shows the strategy for constructing plasmid pTW6165.

20 Figure 10 shows plasmid, pTW6165, encoding the 445 bp synthetic gene for alpha-factor-KPI(-4-57; M15A, S17W) fusion.

25 Figure 11 shows the sequences of the annealed oligonucleotide pairs used to construct plasmids pTW6165, pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, and pTW6174.

Figure 12 shows the sequence of plasmid pTW6166 encoding the fusion of yeast alpha-factor and KPI(-4-57; M15A, S17Y).

30 Figure 13 shows the sequence of plasmid pTW6175 encoding the fusion of yeast alpha-factor and KPI(-4-57; M15L, S17F).

Figure 14 shows the sequence of plasmid pBG028 encoding the fusion of yeast alpha-factor and KPI(-4-57; M15L, S17Y).

35 Figure 15 shows the sequence of plasmid pTW6183 encoding the fusion of yeast alpha-factor and KPI(-4-57; I16H, S17F).

Figure 16 shows the sequence of plasmid PTW6184 encoding the fusion of yeast alpha-factor and KPI(-4-57; I16H, S17Y).

5 Figure 17 shows the sequence of plasmid PTW6185 encoding the fusion of yeast alpha-factor and KPI(-4-57; I16H, S17W).

Figure 18 shows the sequence of plasmid PTW6173 encoding the fusion of yeast alpha-factor and KPI(-4-57; M15A, I16H).

10 Figure 19 shows the sequence of plasmid PTW6174 encoding the fusion of yeast alpha-factor and KPI(-4-57; M15L, I16H).

Figure 20 shows the amino acid sequence of KPI (-4-57; M15A, S17W).

15 Figure 21 shows the amino acid sequence of KPI (-4-57; M15A, S17Y).

Figure 22 shows the amino acid sequence of KPI (-4-57; M15L, S17F).

20 Figure 23 shows the amino acid sequence of KPI (-4-57; M15L, S17Y).

Figure 24 shows the amino acid sequence of KPI (-4-57; I16H, S17F).

Figure 25 shows the amino acid sequence of KPI (-4-57; I16H, S17Y).

25 Figure 26 shows the amino acid sequence of KPI (-4-57; I16H, S17W).

Figure 27 shows the amino acid sequence of KPI (-4-57; M15A, S17F).

30 Figure 28 shows the amino acid sequence of KPI (-4-57; M15A, I16H).

Figure 29 shows the amino acid sequence of KPI (-4-57; M15L, I16H).

Figure 30 shows the construction of plasmid pSP26:Amp:F1.

35 Figure 31 shows the construction of plasmid pgIII.

Figure 32 shows the construction of plasmid pPhoA:KPI:gIII.

Figure 33 shows the construction of plasmid pLG1.

Figure 34 shows the construction of plasmid pAL51.

Figure 35 shows the construction of plasmid pAL53.

Figure 36 shows the construction of plasmid PSP26:Amp:F1:PhoA:KPI:geneIII.

Figure 37 shows the construction of plasmid PDW1 #14.

5 Figure 38 shows the coding region for the fusion of *phoA*-KPI (1-55)-geneIII.

Figure 39 shows the construction of plasmid PDW1 14-2.

10 Figure 40 shows the construction of KPI Library 16-19.

Figure 41 shows the expression unit encoded by the members of KPI Library 16-19.

15 Figure 42 shows the *phoA*-KPI(1-55)-geneIII region encoded by the most frequently occurring randomized KPI region.

Figure 43 shows the construction of pDD185 KPI (-4-57; M15A, S17F).

Figure 44 shows the sequence of alpha-factor fused to KPI (-4-57; M15A, S17F).

20 Figure 45 shows the inhibition constants (K_i) determined for purified KPI variants against the selected serine proteases kallikrein, factor Xa, and factor XIIa.

25 Figure 46 shows the inhibition constants (K_i) determined for KPI variants against kallikrein, plasmin, and factors Xa, XIa, and XIIa.

Figure 47 shows the post-surgical blood loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

30 Figure 48 shows the post-surgical hemoglobin loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 49 shows the oxygen tension in the presence and absence of KPI, before CPB, immediately after CPB, and at 60 and 180 minutes after the end of CPB.

35 Figure 50 summarizes the results shown in Figures 47-49.

Figure 51 shows the sequence of plasmid PTW6166 encoding the fusion of yeast alpha-fact r and KPI(-4-57; M15A, S17Y).

Figure 52 shows the sequence of plasmid PTW6175 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17F).

5 Figure 53 shows the sequence of plasmid PBG028 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17Y).

Figure 54 shows the inhibition constants (K_i) determined for KPI variants against kallikrein, plasmin, and factor XIIa.

10 Detailed Description

The present invention provides peptides that can bind to and preferably inhibit the activity of serine proteases. These inhibitory peptides can also provide a means of ameliorating, treating or preventing clinical
15 conditions associated with increased activity of serine proteases. The novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest than known serine protease inhibitors. Examples
20 of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3;
25 enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

Peptides of the present invention may be used to reduce the tissue damage caused by activation of the proteases of the contact pathway of the blood during
30 surgical procedures such as cardiopulmonary bypass (CPB). Inhibition of contact pathway proteases reduces the "whole body inflammatory response" that can accompany contact pathway activation, and that can lead to tissue damage, and possibly death. The peptides of the present
35 invention may also be used in conjunction with surgical procedures to reduce activated serine protease-associated peri operative and postoperative blood loss. For instance, perioperative blood loss of this type may be

particularly severe during CPB surgery. Pharmaceutical compositions comprising the peptides of the present invention may be used in conjunction with surgery such as CPB; administration of such compositions may occur preoperatively, perioperatively or postoperatively. Examples of other clinical conditions associated with increased serine protease activity for which the peptides of the present invention may be used include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of preferable uses of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The invention is based upon the novel substitution of amino acid residues in the peptide corresponding to the naturally occurring KPI protease inhibitor domain of human amyloid β -amyloid precursor protein (APPI). These substitutions produce peptides that can bind to serine proteases and preferably exhibit an inhibition of the activity of serine proteases. The peptides also preferably exhibit a more potent and specific serine protease inhibition than known serine protease inhibitors. In accordance with the invention, peptides are provided that may exhibit a more potent and specific inhibition of one or more serine proteases of interest, e.g., kallikrein, plasmin and factors Xa, XIa, XIIa, and XIIIa.

The present invention also includes pharmaceutical compositions comprising an effective amount of at least one of the peptides of the invention, in combination with a pharmaceutically acceptable sterile vehicle, as described in REMINGTON'S PHARMACEUTICAL SCIENCES: DRUG RECEPTORS AND RECEPTOR THEORY, (18th ed.), Mack Publishing Co., Easton, PA (1990).

A. Selection of sequences of KPI variants

The sequence of KPI is shown in Table 1. Table 2 shows a comparison of this sequence with that of aprotinin, with which it shares about 45% sequence identity. The numbering convention for KPI shown in Table 1 and used hereinafter designates the first glutamic acid residue of KPI as residue 1. This corresponds to residue number 3 using the standard numbering convention for aprotinin.

The crystal structure for KPI complexed with trypsin has been determined. See Perona et al., *J. Mol. Biol.* 230:919 (1993). The three-dimensional structure reveals two binding loops within KPI that contact the protease. The first loop extends from residue Thr³ to Ile¹⁶, and the second loop extends from residue Phe²³ to Gly²⁷. The two protease binding loops are joined through the disulfide bridge extending from Cys¹² to Cys²⁴. KPI contains two other disulfide bridges, between Cys³ and Cys²³, and between Cys²³ to Cys²⁷.

This structure was used as a guide to inform our strategy for making the amino acid residue substitutions that will be most likely to affect the protease inhibitory properties of KPI. Our examination of the structure indicated that certain amino acid residues, including residues 9, 11, 13-18, 32, and 37-40, appear to be of particular significance in determining the protease binding properties of the KPI peptide. In a preferred embodiment of the invention two or more of those KPI peptide residues are substituted; such substitutions preferably occurring among residues 9, 11, 13-18, 32, and 37-40. In particular, we found that those substituted peptides, including peptides comprising substitutions of at least two of the four residues at positions 15-18, may exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than exhibited by the natural KPI peptide domain. Such substituted peptides may further comprise one or more additional substitutions at residues 9, 11, 13, 14, 32 and 37-40; in particular, such peptides may further

comprise a substitution at positions 9 or 37, or an additional substitution at residue 13. In particular, the peptides of the present invention preferably exhibit a greater potency and specificity for inhibiting one or more serine proteases of interest (e.g., kallikrein, plasmin and factors VIIa, IXa, Xa, XIa, and XIIa) than the potency and specificity exhibited by native KPI or other known serine protease inhibitors. That greater potency and specificity may be manifested by the peptides of the present invention by exhibiting binding constants for serine proteases of interest that are less than the binding constants exhibited by native KPI, or other known serine protease inhibitors, for such proteases.

As an initial guide to informing the choices of amino acid substitution for preparation of KPI variants, the sequences and protease inhibitory activities of aprotinin and KPI are compared. Aprotinin is twice as potent as wild-type KPI with respect to inhibition of human plasma kallikrein, and is 100-fold more potent as an inhibitor of human plasmin. There are three amino acid differences between aprotinin and wild-type KPI in the first protease binding loop extending from residues 9 to 17. A series of KPI variants may then be created, using the methods detailed below, where the residues present in aprotinin at positions 13, 15 and 17 are substituted with the residues found in KPI. The effect of such substitutions upon KPI inhibition of plasma kallikrein and plasmin is then determined.

These results show that substitution of arginine at position 13 by lysine significantly reduces the activity of the resulting protein as an inhibitor of plasma kallikrein. Similarly, substituting positions 15 and 17 of KPI with the corresponding residues found in aprotinin also decreases potency of the KPI variants against kallikrein. Substitutions of aprotinin residues at positions 13 and 15, however, increase the potency of KPI toward plasmin. The single change of methionine to arginine at position 15 (designated M15R) decreases the K_i against plasmin more than 10-fold. The change of

serine to isoleucine at position 17 (S17I) decreases the potency of KPI toward plasmin.

It is observed that single-amino acid substitutions in the first protease binding loop are generally additive, that is, combinations of single amino-acid substitutions, each of which individually enhance the potency toward plasmin, result in variants with even higher potency. The substitution R13K results in a plasmin K_i of 12.3, and the further exchange of M15R results in a K_i that is reduced to 1.45.

It appears, therefore, from these results that combinations of favorable single amino acid substitutions can result in enhanced potency of KPI variants. It is further apparent that substitution in KPI with the residues found in the aprotinin first protease binding loop is not always useful. Although aprotinin is a more potent kallikrein inhibitor than KPI, none of the combinations of aprotinin residues in KPI improve kallikrein inhibition.

To further investigate substitutions that might usefully enhance protease inhibition, a series of single substitutions in KPI is prepared where charged residues in the first protease binding loop are systematically replaced with alanine. This is intended to determine whether substitutions at these sites affect potency toward plasma kallikrein, factor XIIa or plasmin.

It is found that replacement of arginine at position 13 (R13A) drastically reduces KPI inhibition of kallikrein, XIIa or plasmin. The replacement I16A, however, significantly increases the K_i towards both kallikrein and plasmin, suggesting that this amino acid position is critical to inhibition of these proteases. The S17A substitution has little effect. The substitution R18A has little effect upon plasmin inhibition, but significantly impacts inhibition of kallikrein and factor XIIa. These results suggest that substitutions at positions I16 and R18 have the potential to significantly alter the potency of KPI toward kallikrein or plasmin.

These results also suggest that substitutions at residues M15 and S17 could have major effects upon inhibition of kallikrein, XIIa or plasmin. To investigate this further, two sets of yeast expression plasmids are prepared, using the methods described in detail below, in which either M15 or S17 are replaced with all possible amino acids.

Yeast are transformed with these two sets of plasmids, and 100 individual colonies are picked at random from each transformation. Small cultures are grown from each of these colonies, and their conditioned broth is harvested and tested for kallikrein inhibiting activity. The plasmids from colonies yielding cultures expressing KPI variants more potent than wild-type KPI are isolated, and the KPI domain are sequenced. It is found that only four 4 substitutions at position 15: M15A, M15L, M15S, M15V; and 4 substitutions at position 17: S17P, S17F, S17Y and S17W, result in KPI variants with improved potency toward kallikrein.

Combinations of these position 15 and 17 mutants are then prepared to test if their effects on potency of protease inhibition are additive. Four of these double mutants ([M15A, S17Y], [M15A, S17W], [M15L, S17Y] and [M15L, S17P]) are substantially more potent toward kallikrein and factor XIIa than the single amino acid substitutions on which they are based.

The results of changing arginine at positions 18 for alanine also suggest that substitutions at position 18 could affect inhibition of kallikrein and factor XIIa. The KPI double variant M13A, S17W (named TW6165 below) is used to construct a series of variants where all possible amino acid substitutions other than Cys and Arg are placed at position 18. Of these variants, three ([M13A, S17W, R18H], [M13A, S17W, R18Q], and [M13A, S17W, R18T]) are found to exhibit enhanced inhibition of kallikrein and Factor XIIa.

The results described above relate to proteins having the N-terminal sequence EVVREVCN- et seq., as found in KPI (-4-57). The present invention also relates, however

to proteins wherein the N-terminal sequence may be varied, preferably by substituting aspartic acid at the N-terminus in place of the glutamic acid (i.e. the N-terminal sequence is DVVREVCVCS-). Other N-terminal sequences that may be used will be apparent to the skilled artisan, including a sequence lacking the first four amino acids of KPI(-4-57), i.e. having the sequence EVCS-.

By way of example, and as set forth in greater detail below, the serine protease inhibitory properties of peptides of the present invention were measured for the serine proteases of interest — kallikrein, plasmin and factors Xa, XIa, and XIIa. Methodologies for measuring the inhibitory properties of the KPI variants of the present invention are known to those skilled in the art, e.g., by determining the inhibition constants of the variants toward serine proteases of interest, as described in Example 4, *infra*. Such studies measure the ability of the novel peptides of the present invention to bind to one or more serine proteases of interest and to preferably exhibit a greater potency and specificity for inhibiting one or more serine protease of interest than known serine protease inhibitors such as native KPI.

The ability of the peptides of the present invention to bind one or more serine proteases of interest, particularly the ability of the peptides to exhibit such greater potency and specificity toward serine proteases of interest, manifest the clinical and therapeutic applications of such peptides. The clinical and therapeutic efficacy of the peptides of the present invention can be assayed by *in vitro* and *in vivo* methodologies known to those skilled in the art, e.g., as described in Example 5, *infra*.

Table 1: SEQUENCE OF KPI:

1	10	20	30
V R E V C S E Q A E T G P C R A M I S R W Y F D V T E G K C A P			
1	10	20	30
F P Y G G C G G N R N N F D T E E Y C M A V C G S A I			

Table 2: COMPARISON OF KPI AND APROTININ SEQUENCES:

	1	10	20	30	40	50
KPI:	V R E V C S E Q A E T G P C R A M I S R W Y F D V T E G K C A P F E Y G G C G G N R N N F D T E E Y C M A V C G S A I					
BPTI:	R P D F C L E P P Y T G R C K A R I I R Y F Y N A G L C Q T F Y Y G G C R A K R N N F K S A E D C M R T C G G A					
	1	10	20	30	40	50

B. Methods of producing KPI variants

The peptides of the present invention can be created by synthetic techniques or recombinant techniques which employ genomic or cDNA cloning methods.

5 1. Production by chemical synthesis

Peptides of the present invention can be routinely synthesized using solid phase or solution phase peptide synthesis. Methods of preparing relatively short peptides such as KPI by chemical synthesis are well known
10 in the art. KPI variants could, for example be produced by solid-phase peptide synthesis techniques using commercially available equipment and reagents such as those available from Milligen (Bedford, MA) or Applied Biosystems-Perkin Elmer (Foster City, CA).
15 Alternatively, segments of KPI variants could be prepared by solid-phase synthesis and linked together using segment condensation methods such as those described by Dawson et al., Science 266:776 (1994). During chemical
20 synthesis of the KPI variants, substitution of any amino acid is achieved simply by replacement of the residue that is to be substituted with a different amino acid monomer.

2. Production by recombinant DNA technology

25 (a) Preparation of genes encoding KPI variants

In a preferred embodiment of the invention, KPI variants are produced by recombinant DNA technology. This requires the preparation of genes encoding each KPI variant that is to be made. Suitable genes can be
30 constructed by oligonucleotide synthesis using commercially available equipment, such as that provided by Milligen and Applied Biosystems, supra. The genes can be prepared by synthesizing the entire coding and non-coding strands, followed by annealing the two strands.
35 Alternatively, the genes can be prepared by ligation of smaller synthetic oligonucleotides by methods well known in the art. Genes encoding KPI variants are produced by

varying the nucleotides introduced at any step of the synthesis to change the amino acid sequence encoded by the gene.

Preferably, however, KPI variants are made by site-directed mutagenesis of a gene encoding KPI. Methods of site-directed mutagenesis are well known in the art. See, for example, Ausubel et al., (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley Interscience, 1987); PROTEIN ENGINEERING (Oxender & Fox eds., A. Liss, Inc. 1987). These methods require the availability of a gene encoding KPI or a variant thereof, which can then be mutagenized by known methods to produce the desired KPI variants. In addition, linker-scanning and polymerase chain reaction ("PCR") mediated techniques can be used for purposes of mutagenesis. See PCR TECHNOLOGY (Erich ed., Stockton Press 1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, loc. cit.

A gene encoding KPI can be obtained by cloning the naturally occurring gene, as described for example in U.S. Patents Nos. 5,223,482 and 5,187,153, which are hereby incorporated by reference in their entireties. In particular, see columns 6-9 of U.S. Patent No. 5,187,153. See also PCT Application No. 93/09233. In a preferred embodiment of the invention a synthetic gene encoding KPI is produced by chemical synthesis, as described above. The gene may encode the 57-amino acid KPI domain shown in Table 1, or it may also encode additional N-terminal amino acids from the APPI protein sequence, such as the four amino acid sequence (Glu-Val-Val-Arg, designated residues -4 to -1) immediately preceding the KPI domain in APPI.

Production of the gene by synthesis allows the codon usage of the KPI gene to be altered to introduce convenient restriction endonuclease recognition sites, without altering the sequence of the encoded peptide. In a preferred embodiment of the invention, the synthetic KPI gene contains restriction endonuclease recognition sites that facilitate excision of DNA cassettes from the KPI gene. These cassettes can be replaced with small

synthetic oligonucleotides encoding the desired changes in the KPI peptide sequence. See Ausubel, *supra*.

5 This method also allows the production of genes encoding KPI as a fusion peptide with one or more additional peptide or protein sequences. The DNA encoding these additional sequences is arranged in-frame with the sequence encoding KPI such that, upon translation of the gene, a fusion protein of KPI and the additional peptide or protein sequence is produced.
10 Methods of making such fusion proteins are well known in the art. Examples of additional peptide sequences that can be encoded in the genes are secretory signal peptide sequences, such as bacterial leader sequences, for example *ompA* and *phoA*, that direct secretion of proteins to the bacterial periplasmic space. In a preferred
15 embodiment of the invention, the additional peptide sequence is a yeast secretory signal sequence, such as α -mating factor, that directs secretion of the peptide when produced in yeast.

20 Additional genetic regulatory sequences can also be introduced into the synthetic gene that are operably linked to the coding sequence of the gene, thereby allowing synthesis of the protein encoded by the gene when the gene is introduced into a host cell. Examples
25 of regulatory genetic sequences that can be introduced are: promoter and enhancer sequences and transcriptional and translational control sequences. Other regulatory sequences are well known in the art. See Ausubel et al., *supra*, and Sambrook et al., *supra*.

30 Sequences encoding other fusion proteins and genetic elements are well known to those of skill in the art. In a preferred embodiment of the invention, the KPI sequence is prepared by ligating together synthetic oligonucleotides to produce a gene encoding an in-frame
35 fusion protein of yeast α -mating factor with either KPI (1-57) or KPI (-4-57).

The gene constructs prepared as described above are conveniently manipulated in host cells using methods of manipulating recombinant DNA techniques that are well

known in the art. See, for example Sambrook et al.,
MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition,
(Cold Spring Harbor Laboratory Press, Cold Spring Harbor,
NY 1989), and Ausubel, *supra*. In a preferred embodiment
5 of the invention the host cell used for manipulating the
KPI constructs is *E. coli*. For example, the construct
can be ligated into a cloning vector and propagated in *E.*
coli by methods that are well known in the art. Suitable
cloning vectors are described in Sambrook, *supra*, or are
10 commercially available from suppliers such as Promega
(Madison, WI), Stratagene (San Diego, CA) and Life
Technologies (Gaithersburg, MD).

Once a gene construct encoding KPI has been obtained,
genes encoding KPI variants are obtained by manipulating
15 the coding sequence of the construct by standard methods
of site-directed mutagenesis, such as excision and
replacement of small DNA cassettes, as described *supra*.
See Ausubel, *supra*, and Sinha et al., *supra*. See also
U.S. Patent 5,373,090, which is herein incorporated by
20 reference in its entirety. See particularly, columns
4-12 of U.S. Patent 5,272,090. These genes are then used
to produce the KPI variant peptides as described below.

Alternatively, KPI variants can be produced using
phage display methods. See, for example, Dennis et al.
25 *supra*, which is hereby incorporated by reference in its
entirety. See also U.S. Patent Nos. 5,223,409 and
5,403,484, which are hereby also incorporated by
reference in their entireties. In these methods,
libraries of genes encoding variants of KPI are fused in-
30 frame to genes encoding surface proteins of filamentous
phage, and the resulting peptides are expressed
(displayed) on the surface of the phage. The phage are
then screened for the ability to bind, under appropriate
conditions, to serine proteases of interest immobilized
35 on a solid support. Large libraries of phage can be
used, allowing simultaneous screening of the binding
properties of a large number of KPI variants. Phage that
have desirable binding properties are isolated and the
sequences of the genes encoding the corresponding KPI

variants is determined. These genes are then used to produce the KPI variant peptides as described below.

(b) Expression of KPI variant peptides

5 Once genes encoding KPI variants have been prepared, they are inserted into an expression vector and used to produce the recombinant peptide. Suitable expression vectors and corresponding methods of expressing recombinant proteins and peptides are well known in the art. Methods of expressing KPI peptides are described in 10 U.S. Patent 5,187,153, columns 9-11, U.S. Patent 5,223,482, columns 9-11, and PCT application 93/09233, pp. 49-67. See also Ausubel et al., supra, and Sambrook et al., supra. The gene can be expressed in any number 15 of different recombinant DNA expression systems to generate large amounts of the KPI variant, which can then be purified and tested for its ability to bind to and inhibit serine proteases of interest.

 Examples of expression systems known to the skilled practitioner in the art include bacteria such as *E. coli*, 20 yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris*, baculovirus, and mammalian expression systems such as in Cos or CHO cells. In a preferred embodiment, KPI variants are expressed in *Pichia pastoris*. In 25 another preferred embodiment the KPI variants are cloned into expression vectors to produce a chimeric gene encoding a fusion protein of the KPI variant with yeast α -mating factor. The mating factor acts as a signal sequence to direct secretion of the fusion protein from the yeast cell, and is then cleaved from the fusion 30 protein by a membrane-bound protease during the secretion process. The expression vector is transformed into *S. cerevisiae*, the transformed yeast cells are cultured by standard methods, and the KPI variant is purified from 35 the yeast growth medium.

 Recombinant bacterial cells expressing the peptides of the present invention, for example, *E. coli*, are grown in any of a number of suitable media, for example LB, and the expression of the recombinant antigen induced by

adding IPTG to the media or switching incubation to a higher temperature. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a selective speed. If the recombinant peptide is expressed in inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to assist in the removal of any contaminating host proteins, then solubilized in solutions containing high concentrations of urea (e.g., 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents such as β -mercaptoethanol or DTT (dithiothreitol).

At this stage it may be advantageous to incubate the peptides of the present invention for several hours under conditions suitable for the peptides to undergo a refolding process into a conformation which more closely resembles that of native KPI. Such conditions generally include low protein concentrations less than 500 μ g/ml, low levels of reducing agent, concentrations of urea less than 2M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulphide bonds within the protein molecule. The refolding process can be monitored, for example, by SDS-PAGE or with antibodies which are specific for the native molecule (which can be obtained from animals vaccinated with the native molecule isolated from parasites). Following refolding, the peptide can then be purified further and separated from the refolding mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns.

Purification of KPI variants can be achieved by standard methods of protein purification, e.g., using various chromatographic methods including high performance liquid chromatography and adsorption chromatography. The purity and the quality of the peptides can be confirmed by amino acid analyses, molecular weight determination, sequence determination and mass spectrometry. See, for example, PROTEIN PURIFICATION METHODS - A PRACTICAL APPROACH, Harris et al., eds. (IRL Press, Oxford, 1989). In a preferred embodiment, the yeast cells are removed from the growth medium by filtration or centrifugation, and the KPI variant is purified by affinity chromatography on a column of trypsin-agarose, followed by reversed-phase HPLC.

C. Measurement of protease inhibitory properties of KPI variants

Once KPI variants have been purified, they are tested for their ability to bind to and inhibit serine proteases of interest in vitro. The peptides of the present invention preferably exhibit a more potent and specific inhibition of serine proteases of interest than known serine protease inhibitors, such as the natural KPI peptide domain. Such binding and inhibition can be assayed for by determining the inhibition constants for the peptides of the present invention toward serine proteases of interest and comparing those constants with constants determined for known serine protease inhibitors, e.g., the native KPI domain, toward those proteases. Methods for determining inhibition constants of protease inhibitors are well known in the art. See Fersht, ENZYME STRUCTURE AND MECHANISM, 2nd ed., W.H. Freeman and Co., New York, (1985).

In a preferred embodiment the inhibition experiments are carried out using a chromogenic synthetic protease substrate, as described, for example, in Bender et al., J. Amer. Chem. Soc. 88:5890 (1966). Measurements taken by this method can be used to calculate inhibition

constants (K_i values) of the peptides of the present invention toward serine proteases of interest. See Bieth in BAYER-SYMPOSIUM V "PROTEINASE INHIBITORS", Fritz et al., eds., pp. 463-69, Springer-Verlag, Berlin, Heidelberg, New York, (1974). KPI variants that exhibit potent and specific inhibition of one or more serine proteases of interest may subsequently be tested *in vivo*. *In vitro* testing, however, is not a prerequisite for *in vivo* studies of the peptides of the present invention.

10 D. Testing of KPI variants *in vivo*

The peptides of the present invention may be tested, alone or in combination, for their therapeutic efficacy by various *in vivo* methodologies known to those skilled in the art, e.g., the ability of KPI variants to reduce postoperative bleeding can be tested in standard animal models. For example, cardiopulmonary bypass surgery can be carried out on animals such as pigs in the presence of KPI variants, or in control animals where the KPI variant is not used. The use of pigs as a model for studying the clinical effects associated with CPB has previously been described. See Redmond et al., Ann. Thorac. Surg. 56:474 (1993).

25 The KPI variant is supplied to the animals in a pharmaceutical sterile vehicle by methods known in the art, for example by continuous intravenous infusion. Chest tubes can be used to collect shed blood for a defined period of time. The shed blood, together with the residual intrathoracic blood found after sacrifice of the animal can be used to calculate hemoglobin (Hgb) loss. The postoperative blood and Hgb loss is then compared between the test and control animals to determine the effect of the KPI variants.

E. Therapeutic use of KPI variants

35 KPI variants of the present invention found to exhibit therapeutic efficacy (e.g., reduction of blood loss following surgery in animal models) may preferably be used and administered, alone or in combination or as

a fusion protein, in a manner analogous to that currently used for aprotinin or other known serine protease inhibitors. See Butler et al., *supra*. Peptides of the present invention generally may be administered in the manner that natural peptides are administered. A therapeutically effective dose of the peptides of the present invention preferably affects the activity of the serine proteases of interest such that the clinical condition may be treated, ameliorated or prevented. Therapeutically effective dosages of the peptides of the present invention can be determined by those skilled in the art, e.g., through *in vivo* or *in vitro* models. Generally, the peptides of the present invention may be administered in total amounts of approximately 0.01 to approximately 500, specifically 0.1 to 100 mg/kg body weight, if desired in the form of one or more administrations, to achieve therapeutic effect. It may, however, be necessary to deviate from such administration amounts, in particular depending on the nature and body weight of the individual to be treated, the nature of the medical condition to be treated, the type of preparation and the administration of the peptide, and the time interval over which such administration occurs. Thus, it may in some cases be sufficient to use less than the above amount of the peptides of the present invention, while in other cases the above amount is preferably exceeded. The optimal dose required in each case and the type of administration of the peptides of the present invention can be determined by one skilled in the art in view of the circumstances surrounding such administration. Such peptides can be administered by intravenous injections, *in situ* injections, local applications, inhalation, oral administration using coated polymers, dermal patches or other appropriate means. Compositions comprising peptides of the present invention are advantageously administered in the form of injectable compositions. Such peptides may be preferably administered to patients via continuous intravenous infusion, but can also be administered by single or

multiple injections. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described in REMINGTON'S PHARMACEUTICAL SCIENCES, pp. 1405-12 and 1461-87 (1975) and THE NATIONAL FORMULARY XIV., 14th Ed. Washington: American Pharmaceutical Association (1975). Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobials, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components of the composition are adjusted according to routine skills in the art. See GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS FOR THERAPEUTICS (7th ed.). The peptides of the present invention may be present in such pharmaceutical preparations in a concentration of approximately 0.1 to 99.5% by weight, specifically 0.5 to 95% by weight, relative to the total mixture. Such pharmaceutical preparations may also comprise other pharmaceutically active substances in addition to the peptides of the present invention. Other methods of delivering the peptides to patients will be readily apparent to the skilled artisan.

Examples of mammalian serine proteases that may exhibit inhibition by the peptides of the present invention include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as thrombin and factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. Examples of conditions associated with increased serine protease activity include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced

protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of the use of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The inhibitors of the present invention may also be used for inhibition of serine protease activity in vitro, for example during the preparation of cellular extracts to prevent degradation of cellular proteins. For this purpose the inhibitors of the present invention may preferably be used in a manner analogous to the way that aprotinin, or other known serine protease inhibitors, are used. The use of aprotinin as a protease inhibitor for preparation of cellular extracts is well known in the art, and aprotinin is sold commercially for this purpose.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

Example 1. Expression of wild-type KPI (-4-57)

A. Construction of PTW10:KPI

Plasmid PTW10:KPI is a bacterial expression vector encoding the 57 amino acid form of KPI fused to the bacterial *phoA* signal sequence. The strategy for the construction of PTW10:KPI is shown in Figure 1.

Plasmid pcDNAII (Invitrogen, San Diego, CA) was digested with *PvuII* and the larger of the two resulting *PvuII* fragments (3013 bp) was isolated. Bacterial expression plasmid pSP26 was digested with *MluI* and *RsrII*, and the 409 bp *MluI*-*RsrII* fragment containing the pTrp promoter element and transcription termination signals was isolated by electrophoresis in a 3% NuSieve Agarose gel (FMC Corp., Rockland, ME). Plasmid pSP26, containing a heparin-binding EGF-like growth factor (HB-EGF) insert between the *NdeI* and *HindIII* sites, is

described as pNA28 in Thompson et al., *J. Biol. Chem.* 269:2541 (1994). Plasmid pSP26 was deposited in host *E. coli* W3110, pSP26 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, USA under the conditions specified by the Budapest Treaty on the International Recognition of the Deposit of Microorganisms (Budapest Treaty). Host *E. coli* W3110, pSP26 was deposited on 3 May 1995 and given Accession No. 69800. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The ends of the *Mlu*I-*Rsr*II fragment were blunted using DNA polymerase Klenow fragment by standard techniques. The blunted fragment of pSP26 was then ligated into the large *Pvu*II fragment of plasmid pCDNAII, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to isolate plasmid pTW10 by standard techniques.

A synthetic gene was constructed encoding the bacterial *phoA* secretory signal sequence fused to the amino terminus of KPI(1-57). The synthetic gene contains cohesive ends for *Nde*I and *Hind*III, and also incorporates restriction endonuclease recognition sites for *Age*I, *Rsr*II, *Aat*II and *Bam*HI, as shown in Figure 2. The synthetic *phoA*-KPI gene was constructed from 6 oligonucleotides of the following sequences (shown 5'→3'):

6167:

TATGAAACAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCC
CTGTGACAAAGCCGAGGTGTGCTCTGAA

6169:

CTCGGCTTTTGTACAGGGGTAAACAGTAACGGTAAGAGTGCCAGTGCAA
TAGTGCTTTGTTTCATA

6165:

CAAGCTGAGACCGGTCCGTGCCGTGCAATGATCTCCCGCTGGTACTTTGA
CGTCACTGAAGGTAAGTGGCTCCATTCTTT

6166:
GCACTTACCTTCAGTGACGTCAAAGTACCAGCGGGAGATCATTGCACGGC
ACGGACCGGTCTCAGCTTGTTTCAGAGCACAC

5 6168:
TACGGCGGTTGCGGCGGCAACCGTAACAACTTTGACACTGAAGAGTACTG
CATGGCAGTGTGCGGATCCGCTATTTAAGCT

6164:
AGCTTAAATAGCGGATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAA
AGTTGTTACGGTTGCCGCGCAACCGCCGTAAAAGAATGGAGC

10 The oligonucleotides were phosphorylated and annealed
in pairs: 6167 + 6169, 6165 + 6166, 6168 + 6164. In
20 μ l T4 DNA Ligase Buffer (New England Biolabs,
Beverley, MA), 1 μ g of each oligonucleotide pair was
incubated with 10 U T4 Polynucleotide Kinase (New England
15 Biolabs) for 1 h at 37°C, then heated to 95°C for 1
minute, and slow-cooled to room temperature to allow
annealing. All three annealed oligo pairs were then
mixed for ligation to one another in a total volume of
100 μ l T4 DNA Ligase Buffer, and incubated with 400 U T4
20 DNA Ligase (New England Biolabs) overnight at 15°C. The
ligation mixture was extracted with an equal volume of
phenol:CHCl₃ (1:1), ethanol-precipitated, resuspended in
50 μ l Restriction Endonuclease Buffer #4 (New England
Biolabs) and digested with *Nde*I and *Hind*III. The
25 annealed, ligated and digested oligos were then subjected
to electrophoresis in a 3% NuSieve Agarose gel, and the
240 bp *Nde*I-*Hind*III fragment was excised. This gel-
purified synthetic gene was ligated into plasmid pTW10
which had previously been digested with *Nde*I and *Hind*III,
30 and the ligation mixture was used to transform *E. coli*
strain MC1061. Ampicillin-resistant colonies were
selected and used to prepare plasmid pTW10:KPI. This
plasmid contains the *phoA*-KPI(1-57) fusion protein
inserted between the pTrp promoter element and the
35 transcription termination signals.

B. Construction of pKPI-61

The strategy for constructing pKPI-61 is shown in
Figure 3. Plasmid pTW10:KPI was digested with *Age*I and

*Hind*III; the resulting 152 bp *Age*I-*Hind*III fragment containing a portion of the KPI synthetic gene was isolated by preparative gel electrophoresis. An oligonucleotide pair (129 + 130) encoding the 9 amino-terminal residues of KPI(1-57) and 4 amino acids of yeast α -mating factor was phosphorylated and annealed as described above.

129: CTAGATAAAAGAGAGGTGTGCTCTGAACAAGCTGAGA
130: CCGGTCTCAGCTTGTTTCAGAGCACACCTCTCTTTTAT

10 The annealed oligonucleotides were then ligated to the *Age*I-*Hind*III fragment of the KPI (1-57) synthetic gene. The resulting 192 bp *Xba*I-*Hind*III synthetic gene (shown in Figure 4) was purified by preparative gel electrophoresis, and ligated into plasmid pUC19 which had
15 previously been digested with *Xba*I and *Hind*III. The ligation products were used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were picked and used to prepare plasmid PKPI-57 by standard methods. To create a synthetic gene encoding KPI(-4-57), PKPI-57 was
20 digested with *Xba*I and *Age*I and the smaller fragment replaced with annealed oligos 234 + 235, which encode 4 amino acid residues of yeast α -mating factor fused a 4 amino acid residue amino-terminal extension of KPI(1-57).

234: CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCTGAGA
25 235: CCGGTCTCAGCTTGTTTCAGAGCACACCTCTCTAACACCTCTCTTTTAT

The 4 extra amino acids are encoded in the amyloid β -protein precursor/protease nexin-2 (APPI) which contains the KPI domain. The synthetic 201 bp *Xba*I-*Hind*III fragment encoding KPI(-4-57) in pKPI-61 is shown
30 in Figure 5.

C. Assembly of pTW113

The strategy for the construction of pTW113 is shown in Figure 6. Plasmid pSP35 was constructed from yeast expression plasmid pYES2 (Invitrogen, San Diego, CA) as

follows. A 267 bp *PvuII*-*XbaI* fragment was generated by PCR from yeast α -mating factor DNA using oligos 6274 and 6273:

6274: GGGGGCAGCTGTATAAACGATTAAAA
 5 6273: GGGGGTCTAGAGATACCCCTTCTTCTTTAG

This PCR fragment, encoding an 82 amino acid portion of yeast α -mating factor, including the secretory signal peptide and pro-region, was inserted into pYES2 that had been previously digested with *PvuII* and *XbaI*. The resulting plasmid is denoted pSP34.

Two oligonucleotide pairs, 6294 + 6292 were then ligated to 6290 + 6291, and the resulting 135 bp fragment was purified by gel electrophoresis.

6294: CTAGATAAAAGAGAGGCTGAGGCTCAOGCTGAAGGTACTTTCACTTC
 15 6290: TGACGTCTCTTCTTACTTGGAAGGTCAAGCTGCTAAGGAATTCAT
 CGCTTGGTTGGTCAAAGGTAGAGGTTAAGCTTA
 6291: CTAGTAAGCTTAACCTCTACCTTTGACCAACCAAGCGATGAATTC
 CTTAGCA
 20 6292: GCTTGACCTTCCAAGTAAGAAGAGACGTCAGAAGTGAAAGTACCT
 TCAGCGTGAGCCTCAGCCTCTCTTTTAT

The resulting synthetic fragment was ligated into the *XbaI* site of pSP34, resulting in plasmid pSP35. pSP35 was digested with *XbaI* and *HindIII* to remove the insert, and ligated with the 201 bp *XbaI*-*HindIII* fragment of pKPI-61, encoding KPI(-4-57). The resulting plasmid pTW113, encodes the 445 bp synthetic gene for the α -factor-KPI(-4-57) fusion. See Figure 7.

D. Transformation of yeast with pTW113

Saccharomyces cerevisiae strain ABL115 was transformed with plasmid pTW113 by electroporation by the method of Becker et al., *Methods Enzymol.* 194:182 (1991). An overnight culture of yeast strain ABL115 was used to inoculate 200 ml YPD medium. The inoculated culture was grown with vigorous shaking at 30°C to an OD₆₀₀ of 1.3-1.5.

at which time the cells were harvested by centrifugation at 5000 rpm for 5 minutes. The cell pellet was resuspended in 200 ml ice-cold water, respun, resuspended in 100 ml ice-cold water, then pelleted again. The washed cell pellet was resuspended in 10 ml ice-cold 1M sorbitol, recentrifuged, then resuspended in a final volume of 0.2 ml ice-cold 1M sorbitol. A 40 μ l aliquot of cells was placed into the chamber of a cold 0.2 cm electroporation cuvette (Invitrogen), along with 100 ng plasmid DNA for pTW113. The cuvette was placed into an
10 Invitrogen Electroporator II and pulsed at 1500 V, 25 μ F, 100 Ω . Electroporated cells were diluted with 0.5 ml 1M sorbitol, and 0.25 ml was spread on an SD agar plate containing 1M sorbitol. After 3 days' growth at 30°C,
15 individual colonies were streaked on SD + CAA agar plates.

E. Induction of pTW113/ABL115, purification of KPI(-4-57)

Yeast cultures were grown in a rich broth and the
20 galactose promoter of the KPI expression vector induced with the addition of galactose as described by Sherman, *Methods Enzymol.* 194:3 (1991). A single well-isolated colony of pTW113/ABL115 was used to inoculate a 10 ml overnight culture in Yeast Batch Medium. The next day,
25 1L Yeast Batch Medium which had been made 0.2% glucose was inoculated to an OD₆₀₀ of 0.1 with the overnight culture. Following 24 hours at 30°C with vigorous shaking, the 1L culture was induced by the addition of 20 ml Yeast Galactose Feed Medium. Following induction, the
30 culture was fed every 12 hours with the addition of 20 ml Yeast Galactose Feed Medium. At 48 hours after induction, the yeast broth was harvested by centrifugation, then adjusted to pH 7.0 with 2M Tris, pH 10. The broth was subjected to trypsin-Sepharose
35 affinity chromatography, and bound KPI(-4-57) was eluted with 20mM Tris pH 2.5. See Schilling et al., *Gene* 98:225 (1991). Final purification of KPI(-4-57) was accomplished by HPLC chromatography on a semi-prep Vydac

C4 column in a gradient of 20% to 35% acetonitrile. The sample was dried and resuspended in PBS at 1-2 mg/ml. The amino acid sequence of KPI(-4-57) is shown in Figure 8.

5 **Example 2. Recombinant Expression of site-directed KPI(-4-57) variants**

Expression vectors for the production of specific variants of KPI(-4-57) were all constructed using the pTW113 backbone as a starting point. For each KPI variant, an expression construct was created by replacing
10 the 40 bp RsrII-AatII fragment of the synthetic KPI gene contained in pTW113 with a pair of annealed oligonucleotides which encode specific codons mutated from the wild-type KPI(-4-57) sequence. In the following
15 Examples the convention used for designating the amino substituents in the KPI variants indicates first the single letter code for the amino acid found in wild-type KPI, followed by the position of the residue using the numbering convention described supra, followed by the
20 code for the replacement amino acid. Thus, for example, M15R indicates that the methionine residue at position 15 is replaced by an arginine.

A. Construction of pTW6165

The strategy for constructing pTW6165 is shown in
25 Figure 9. Plasmid pTW113 was digested with RsrII and AatII, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (812 + 813) was phosphorylated, annealed and gel-purified as described above.

30 812: GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT
813: CAAAGTACCAGCGCCAGATAGCTGCACGGCACC

The annealed oligonucleotides were ligated into the RsrII and AatII-digested pTW113, and the ligation product was used to transform *E. coli* strain MC1061. Transformed
35 colonies were selected by ampicillin resistance. The

resulting plasmid, pTW6165, encodes the 445 bp synthetic gene for the α -factor-KPI(-4 \rightarrow 57; M15A, S17W) fusion. See Figure 10.

5 B. Construction of pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, pTW6174.

Construction of the following KPI(-4 \rightarrow 57) variants was accomplished exactly as outlined for pTW6165. The oligonucleotides utilized for each construct are denoted below, and the sequences of annealed oligonucleotide
10 pairs are shown in Figure 11. Figures 12-19 show the synthetic genes for the α -factor fusions with each KPI(-4 \rightarrow 57) variant.

pTW6166: KPI(-4 \rightarrow 57; M15A, S17Y) — See Figure 12

814: GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT

15 815: CAAAGTACCAGCGGTAGATAGCTGCACGGCAGC

pTW6175: KPI(-4 \rightarrow 57; M15L, S17F) — See Figure 13

867: GTCCGTGCCGTGCATTGATCTTCCGCTGGTACTTTGACGT

868: CAAAGTACCAGCGGAAGATCAATGCACGGCAGC

pBG028: KPI(-4 \rightarrow 57; M15L, S17Y) — See Figure 14

20 1493: GTCCGTGCCGTGCTTTGATCTACCGCTGGTACTTTGACGT

1494: CAAAGTACCAGCGGTAGATCAAAGCACGGCAGC

pTW6183: KPI(-4 \rightarrow 57; I16H, S17F) — See Figure 15

925: GTCCGTGCCGTGCAATGCACCTTCCGCTGGTACTTTGACGT

926: CAAAGTACCAGCGGAAGTGCAATGCACGGCAGC

25 pTW6184: KPI(-4 \rightarrow 57; I16H, S17Y) — See Figure 16

927: GTCCGTGCCGTGCAATGCACCTACCGCTGGTACTTTGACGT

928: CAAAGTACCAGCGGTAGTGCATTGCACGGCAGC

pTW6185: KPI(-4→57; I16H, S17W) — See Figure 17

929: GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT

930: CAAAGTACCAGCGCCAGTGCATTGCACGGCAGC

5 pTW6173: KPI(-4→57; M15A, I16H) — See Figure 18

863: GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT

864: CAAAGTACCAGCGGGAGTGAGCTGCACGGCAGC

pTW6174: KPI(-4→57; M15L, I16H) — See Figure 19

865: GTCCGTGCCGTGCATTGCACTCCCGCTGGTACTTTGACGT

10 866: CAAAGTACCAGCGGGAGTGCAATGCACGGCAGC

C. Transformation of yeast with expression vectors

Yeast strain ABL115 was transformed by electroporation exactly according to the protocol described for transformation by pTW113.

15 D. Induction of transformed yeast strains, purification of KPI(-4→57) variants.

Cultures of yeast strains were grown and induced, and recombinant secreted KPI(-4→57) variants were purified according to the procedure described for KPI(-4→57). The
20 amino acid sequences of KPI(-4→57) variants are shown in Figures 20-29.

Example 3. Identification of KPI (-4→57; M15A, S17F) DD185 by phage display.

A. Construction of vector pSP26:Amp:F1

25 The construction of pSP26:Amp:F1 is outlined in Figure 30. Vector pSP26:Amp:F1 contributes the basic plasmid backbone for the construction of the phage display vector for the phoA:KPI fusion, PDW1 #14. pSP26:Amp:F1 contains a low-copy number origin of

replication, the ampicillin-resistance gene (Amp) and the F1 origin for production of single-stranded phagemid DNA.

The ampicillin-resistance gene (Amp) was generated through polymerase chain reaction (PCR) amplification from the plasmid genome of PUC19 using oligonucleotides 176 and 177.

176: GCCATCGATGGTTTCTTAAGCGTCAGGTGGCACTTTTC
177: GCGCCAATTCTTGGTCTACGGGGTCTGACGCTCAGTGGAAACGAA

The PCR amplification of Amp was done according to standard techniques, using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification from plasmid pUC19 with these oligonucleotides yielded a fragment of 1159 bp, containing PflMI and ClaI restriction sites. The PCR product was digested with PflMI and ClaI and purified by agarose gel electrophoresis in 3% NuSieve Agarose (FMC Corp.). Bacterial expression vector pSP26 (*supra*) was digested with PflMI and ClaI and the larger vector fragment was purified. The PflMI-ClaI PCR fragment was ligated into the previously digested pSP26 containing the Amp gene. The ligation product was used to transform *E. coli* strain MC1061 and colonies were selected by ampicillin resistance. The resulting plasmid is denoted pSP26:Amp.

The F1 origin of replication from the mammalian expression vector pcDNAII (Invitrogen) was isolated in a 692 bp *E*arI fragment. Plasmid pcDNAII was digested with *E*arI and the resulting 692 bp fragment purified by agarose gel electrophoresis. *E*arI-NotI adapters were added to the 692 bp *E*arI fragment by ligation of two annealed oligonucleotide pairs, 179 + 180 and 181 + 182. The oligo pairs were annealed as described above.

179: GGCCGCTCTTCC
180: AAAGGAAGAGC
181: CTAGAATTGC
182: GGCCGCAATTC

The oligonucleotide-ligated fragment was then ligated into the single *NotI* site of PSP26:Amp to yield the vector pPSP26:Amp:F1.

B. Construction of vector pgIII

5 The construction of pgIII is outlined in Figure 31. The portion of the phage geneIII protein gene contained by the PDW1 #14 phagemid vector was originally obtained as a PCR amplification product from vector m13mp8. A portion of m13mp8 geneIII encoding the carboxyl-terminal
10 158 amino acid residues of the geneIII product was isolated by PCR amplification of m13mp8 nucleotide residues 2307-2781 using PCR oligos 6162 and 6160.

6162: GCCGGATCCGCTATTTCCGGTGGTGGCTCTGGTTCC

6160: GCCAAGCTTATTAAAGACTCCTTATTACGCAG

15 The PCR oligos contain *BamHI* and *HindIII* restriction recognition sites such that PCR from m13mp8 plasmid DNA with the oligo pair yielded a 490 bp *BamHI*-*HindIII* fragment encoding the appropriate portion of geneIII. The PCR product was ligated between the *BamHI* and *HindIII*
20 sites within the polylinker of PUC19 to yield plasmid pgIII.

C. Construction of pPhoA:KPI:gIII

Construction of pPhoA:KPI:gIII is outlined in Figure 32. A portion of the phoA signal sequence and KPI
25 fusion encoded by the phage display vector PDW1 #14 originates with pPhoA:KPI:gIII. The 237 bp *NdeI*-*HindIII* fragment of pTW10:KPI encoding the entire phoA:KPI (1-57) fusion was isolated by preparative agarose gel electrophoresis, and inserted between the *NdeI* and
30 *HindIII* sites of pUC19 to yield plasmid pPhoA:KPI. The 490 bp *BamHI*-*HindIII* fragment of pgIII encoding the C-terminal portion of the geneIII product was then isolated and ligated between the *BamHI* and *HindIII* sites of pPhoA:KPI to yield vector pPhoA:KPI:gIII. The
35 pPhoA:KPI:gIII vector encodes a 236 amino acid residue

fusion of the *phoA* signal peptide, KPI (1-57) and the carboxyl-terminal portion of the *geneIII* product.

D. Construction of pLG1

Construction of pLG1 is illustrated in Figure 33. The exact *geneIII* sequences contained in vector PDW1 #14 originate with phage display vector pLG1. A modified *geneIII* segment was generated by PCR amplification of the *geneIII* region from pgIII using PCR oligonucleotides 6308 and 6305.

6308: AGCTCCGATCTAGGATCCGGTGGTGGCTCTGGTTCCGGT
6305: GCAGCGGCCGTTAAGCTTATTAAGACTCCT

PCR amplification from pgIII with these oligonucleotides yielded a 481 bp *Bam*HI-*Hind*III fragment encoding a *geneIII* product shortened by 3 amino acid residues at the amino-terminal portion of the segment of the *geneIII* fragment encoded by pgIII. A 161 bp *Nde*I-*Bam*HI fragment was generated by PCR amplification from bacterial expression plasmid pTHW05 using oligonucleotides 6306 and 6307.

6306: GATCCTTGTCCTCATATGAAACAAAGC
6307: CACGTCGGTCGAGGATCCCTAACCACGGCCTTTAACCAG

The 161 bp *Nde*I-*Bam*HI fragment and the 481 bp *Bam*HI-*Hind*III fragment were gel-purified, and then ligated in a three-way ligation into PTW10 which had previously been digested with *Nde*I and *Hind*III. The resulting plasmid pLG1 encodes a *phoA* signal peptide-insert-*geneIII* fusion for phage display purposes.

E. Construction of pAL51

Construction of pAL51 is illustrated in Figure 34. Vector pAL51 contains the geneIII sequences of pLG1 which are to be incorporated in vector pDW1 #14.

5 A 1693 bp fragment of plasmid pBR322 was isolated, extending from the *Bam*HI site at nucleotide 375 to the *Pvu*II site at position 2064. Plasmid pLG1 was digested with *Asp*718I and *Bam*HI, removing an 87 bp fragment. The overhanging *Asp*718I end was blunted by treatment with
10 Klenow fragment, and the *Pvu*II-*Bam*HI fragment isolated from pBR322 was ligated into this vector, resulting in the insertion of a 1693 bp "stuffer" region between the *Asp*718I and *Bam*HI sites. The 78 bp *Nde*I-*Asp*718I region of the resulting plasmid was removed and replaced with
15 the annealed oligo pair 6512 + 6513.

6512: TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTT
TACCCCGGTGACCAAAGCCACGCTGAAG

6513: GTACCTTCAGCGTGGGCTTTGGTCACCGGGGTAAACAGTAACGGT
AAGAGTGCCAGTGCAATAGTGCTTTGTTTCA

20 The newly created 74 bp *Nde*I-*Asp*718I fragment encodes the *phoA* signal peptide, and contains a *Bst*BII cloning site. The resulting plasmid is denoted pAL51.

F. Construction of pAL53

Construction of pAL53 is outlined in Figure 35. Plasmid pAL53 contributes most of the vector sequence of
25 pDW1 #14, including the basic vector backbone with *Amp* gene, *F1* origin, low copy number origin of replication, geneIII segment, *phoA* promoter and *phoA* signal sequence.

Plasmid pAL51 was digested with *Nde*I and *Hind*III and the resulting 2248 bp *Nde*I-*Hind*III fragment encoding the
30 *phoA* signal peptide, stuffer region and geneIII region was isolated by preparative agarose gel electrophoresis. The *Nde*I-*Hind*III fragment was ligated into plasmid pSP26:*Amp*:*F1* between the *Nde*I and *Hind*III sites,
35 resulting in plasmid pAL52.

The *phoA* promoter region and signal peptide was generated by amplification of a portion of the *E. coli* genome by PCR, using oligonucleotide primers 405 and 406.

405: CCGGACGCGTGGAGATTATCGTCACTG
 5 406: GCTTTGGTCACCGGGGTAAACAGTAACGG

The resulting PCR product is a 332 bp *MluI*-*BstEII* fragment which contains the *phoA* promoter region and signal peptide sequence. This fragment was used to replace the 148 bp *MluI*-*BstEII* segment of PAL52, resulting in vector pAL53.

G. Construction of pSP26:Amp:F1:PhoA:KPI:gIII

Construction of pSP26:Amp:F1:PhoA:KPI:gIII is illustrated in Figure 36. This particular vector is the source of the KPI coding sequence found in vector pDW1 #14. Plasmid pPhoA:KPI:gIII was digested with *NdeI* and *HindIII*, and the resulting 714 bp *NdeI*-*HindIII* fragment was purified, and then inserted into vector pSP26:Amp:F1 between the *NdeI* and *HindIII* sites. The resulting plasmid is denoted pSP26:Amp:F1:PhoA:KPI:gIII.

H. Construction of pDW1 #14

Construction of pDW1 #14 is illustrated in Figure 37. The sequences encoding KPI were amplified from plasmid pSP26:Amp:F1:PhoA:KPI:gIII by PCR, using oligonucleotide primers 424 and 425.

424: CTGTTTACCCCGGTGACCAAAGCCGAGGTGTGCTCTGAACAA
 425: AATAGCGGATCCGCACACTGCCATGCAGTACTCTTC

The resulting 172 bp *BstEII*-*BamHI* fragment encodes most of KPI (1-55). This fragment was used to replace the stuffer region in pAL53 between the *BstEII* and *BamHI* sites. The resulting plasmid, pDW1 #14, is the parent KPI phage display vector for preparation of randomized KPI phage libraries. The coding region for the *phoA*-KPI (1-55)-geneIII fusion is shown in Figure 38.

I. Construction of pDW1 14-2

Construction of pDW1 14-2 is illustrated in Figure 39. The first step in the construction of the KPI phage libraries in pDW1 #14 was the replacement of the AgeI-BamHI fragment within the KPI coding sequence with a stuffer fragment. This greatly aids in preparation of randomized KPI libraries which are substantially free of contamination of phagemid genomes encoding wild-type KPI sequence.

Plasmid pDW1 #14 was digested with AgeI and BamHI, and the 135 bp AgeI-BamHI fragment encoding KPI was discarded. A stuffer fragment was created by PCR amplification of a portion of the PBR322 Tet gene, extending from the BamHI site at nucleotide 375 to nucleotide 1284, using oligo primers 266 and 252.

266: GCTTTAAACCGGTAGGTGGCCCGGCTCCATGCACC
252: CGAATTCACCGGTGTCATCCTCGGCACCGTCACCCT

The resulting 894 bp AgeI-BamHI stuffer fragment was then inserted into the AgeI/BamHI-digested pDW1 #14 to yield the phagemid vector pDW1 14-2. This vector was the starting point for construction of the randomized KPI libraries.

J. Construction of KPI Library 16-19

Construction of KPI Library 16-19 is outlined in Figure 40. Library 16-19 was constructed to display KPI-geneIII fusions in which amino acid positions Ala¹⁴, Met¹⁵, Ile¹⁶ and Ser¹⁷ are randomized. For preparation of the library, plasmid pDW1 14-2 was digested with AgeI and BamHI to remove the stuffer region, and the resulting vector was purified by preparative agarose gel electrophoresis. Plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the AgeI site to the BamHI site. The oligonucleotide primers used were 544 and 551.

544: GGGCTGAGACCGGTCCGTGCCGT(NNS),CGCTGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 544 contains four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop codon, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp AgeI-BamHI fragments all containing different sequences in the randomized region. The PCR product was purified by preparative agarose gel electrophoresis and ligated into the AgeI/BamHI digested PDW1 14-2 vector. The ligation mixture was used to transform *E. coli* Top10F' cells (Invitrogen) by electroporation according to the manufacturer's directions. The resulting Library 16-19 contained approximately 400,000 independent clones. The potential size of the library, based upon the degeneracy of the priming PCR oligo #544 was 1,048,576 members. The expression unit encoded by the members of Library 16-19 is shown in Figure 41.

K. Selection of Library 16-19 with human plasma kallikrein

KPI phage were prepared and amplified by infecting transformed cells with M13KO7 helper phage as described by Matthews et al., *Science* 260:1113 (1993). Human plasma kallikrein (Enzyme Research Laboratories, South Bend, IN), was coupled to Sepharose 6B resin. Prior to phage binding, the immobilized kallikrein resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, CaCl₂, MgCl₂, 0.1% gelatin, and 0.05% Triton X-100). Approximately 5x10⁹ phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 µl kallikrein resin containing 15 pmoles of active human plasma kallikrein in a total volume of 250 µl. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the kallikrein resin three times

in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 5.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titrated and amplified for reselection. After three rounds of selection on kallikrein-Sepharose, phagemid DNA was isolated from 22 individual colonies and subjected to DNA sequence analysis.

The most frequently occurring randomized KPI region encoded: Ala¹⁴-Ala¹⁵-Ile¹⁶-Phe¹⁷. The phoA-KPI-geneIII region encoded by this class of selected KPI phage is shown in Figure 42. The KPI variant encoded by these phagemids is denoted KPI (1-55; M15A, S17F).

L. Construction of pDD185 KPI (-4-57; M15A, S17F)

Figure 43 outlines the construction of pDD185 KPI (-4-57; M15A, S17F). The sequences encoding KPI (1-55; M15A, S17F) were moved from one phagemid vector, pDW1 (16-19) 185, to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4-57) was digested with AgeI and BamHI and the 135 bp AgeI-BamHI fragment was discarded. The 135 bp AgeI-BamHI fragment of pDW1 (16-19) 185 was isolated and ligated into the yeast vector to yield plasmid pDD185, encoding α -factor fused to KPI (-4-57; M15A, S17F). See Figure 44.

M. Purification of KPI (-4-57; M15A, S17F) pDD185

Transformation of yeast strain ABL115 with pDD185, induction of yeast cultures, and purification of KPI (-4-57; M15A, S17F) pDD185 was accomplished as described for the other KPI variants.

N. Construction of KPI Library 6 - M15A, with residues 14, 16-18 random.

Library 6 was constructed to display KPI-geneIII fusions in which amino acid positions Ala¹⁴, Ile¹⁶, Ser¹⁷

and Arg¹⁸ are randomized, but position 15 was held constant as Ala. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the AgeI site to the BamHI site. The oligonucleotide primers used were 551 and 1003.

1003: GCTGAGACCGGTCCGTGCCGTTNNSGCA(NNS),TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1003 contained four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp AgeI-BamHI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with BamHI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with BamHI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with AgeI to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately 5×10^6 independent clones.

O. Construction of KPI Library 7 - residues 14-18 random.

Library 7 was constructed to display KPI-geneIII fusions in which amino acid positions Ala¹⁴, Met¹⁵, Ile¹⁶, Ser¹⁷ and Arg¹⁸ are randomized. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the AgeI site to the BamHI site. The oligonucleotide primers used were 551 and 1179.

1179: GCTGAGACCGGTCCGTGCCGT (NNS), TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1179 contains five randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encoded all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp AgeI-BamHI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with BamHI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with BamHI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with AgeI to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately 1×10^7 independent clones.

P. Selection of Libraries 6 & 7 with human factor XIIa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor XIIa (Enzyme Research Laboratories, South Bend, IN), was biotinylated as follows. Factor XIIa (0.5 mg) in 5mM sodium acetate pH 8.3 was incubated with Biotin Ester (Zymed) at room temperature for 1.5 h, then buffer-exchanged into assay buffer (AB). Approximately 1×10^{10} phage particles of each amplified Library 6 or 7 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were incubated with 50 pmoles of active biotinylated human factor XIIa in a total volume of 200 μ l. Phage were allowed to bind for 2 h at room temperature, with rocking. Following the binding period, 100 μ l Streptavidin Magnetic Particles (Boehringer

Mannheim) were added to the mixture and incubated at room temperature for 30 minutes. Separation of magnetic particles from the supernatant and wash/elution buffers was carried out using MPC-E-1 Neodymium-iron-boron permanent magnets (Dyna). Unbound phage were removed by washing the magnetically bound biotinylated XIIa-phage complexes three times with 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After 3 or 4 rounds of selection with factor XIIa, phagemid DNA was isolated from individual colonies and subjected to DNA sequence analysis.

Sequences in the randomized regions were compared with one another to identify consensus sequences appearing more than once. From Library 6 a phagemid was identified which encoded M15L, S17Y, R18H. From Library 7 a phagemid was identified which encoded M15A, S17Y, R18H.

Q. Construction of pBG015 KPI (-4-57; M15L, S17Y, R18H), pBG022 (-4-57; M15A, S17Y, R18H)

The sequences encoding KPI (1-55; M15L, S17Y, R18H) and KPI (1-55; M17A, S17Y, R18H) were moved from the phagemid vectors to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4-57) was digested with AgeI and BamHI and the 135 bp AgeI-BamHI fragment was discarded. The 135 bp AgeI-BamHI fragment of the phagemid vectors were isolated and ligated into the yeast vector to yield plasmids pBG015 and pBG022, encoding alpha-factor fused to KPI (-4-57; M15L, S17Y, R18H), and KPI (-4-57; M15A, S17Y, R18H), respectively.

R. Construction of pBG029 KPI (-4→57, T9V, M15L, S17Y, R18H)

Plasmid pBG015 was digested with XbaI and RsrII, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously.

1593: CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCT
GAGGTTG

1642: GACCAACCTCAGCTTGTTCAGAGCACACCTCTCTAA
CAACCTCTCTTTTAT

The annealed oligonucleotides were ligated into the XbaI and RsrII-digested pBG015, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG029, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15L, S17F, R18H) fusion.

S. Construction of pBG033 KPI (-4→57; T9V, M15A, S17Y, R18H)

Plasmid pBG022 was digested with XbaI and RsrII, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously. The annealed oligonucleotides were ligated into the XbaI and RsrII-digested pBG022, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG033, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15A, S17F, R18H) fusion.

T. Selection of Library 16-19 with human factor Xa

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor Xa (Haematologic Technologies, Inc., Essex Junction, VT) was coupled to Sepharose 6B resin. Prior to phage binding, the

immobilized Xa resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, CaCl₂, MgCl₂, 0.1% gelatin, and 0.05% Triton X-100). Approximately 4×10^{10} phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 μ l Xa resin in a total volume of 250 μ l. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the Xa resin three times in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on Xa-Sepharose, phagemid DNA was isolated and subjected to DNA sequence analysis.

Sequences in the randomized Ala¹⁴-Ser¹⁷ region were compared with one another to identify consensus sequences appearing more than once. A phagemid was identified which encoded KPI (1-55; M15L, I16F, S17K).

U. Construction of pDD131 KPI (-4-57; M15L, I16F, S17K)

The sequences encoding KPI (1-55; M15L, I16F, S17K) were moved from the phagemid vector to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4-57) was digested with AgeI and BamHI and the 135 bp AgeI-BamHI fragment was discarded. The 135 bp AgeI-BamHI fragment of the phagemid vector was isolated and ligated into the yeast vector to yield plasmid pDD131, encoding alpha-factor fused to KPI (-4-57; M15L, I16F, S17K).

V. Construction of pDD134 KPI (-4→57; M15L, I16F, S17K, G37Y)

Plasmid pDD131 was digested with AatI and BamHI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (738 + 739) was phosphorylated, annealed and gel-purified as described previously.

738: CACTGAAGGTAAGTGGCTCCATTCTTTTACGGCGGTTGCTACGGCAACCGT
AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739: GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the AatI and BamHI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD134, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; M15L, I16F, S17K, G37Y) fusion.

W. Construction of pDD135 KPI (-4→57; M15L, I16F, S17K, G37L)

Plasmid pDD131 was digested with AatII and BamHI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (724 + 725) was phosphorylated, annealed and gel-purified as described previously.

738: CACTGAAGGTAAGTGGCTCCATTCTTTTACGGCGGTTGCTACGGCAACCGT
AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739: GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the AatII and BamHI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD135, encodes the

445 bp synthetic gene for the alpha-factor-KPI (-4-57; M15L, I16F, S17K, G37L) fusion.

Example 4. Kinetic analysis of KPI(-4-57) variants

The concentrations of active human plasma kallikrein, factor XIIa, and trypsin were determined by titration with p-nitrophenyl p'-guanidinobenzoate as described by Bender et al., *supra*, and Chase et al., *Biochem. Biophys. Res. Commun.* 29:508 (1967). Accurate concentrations of active KPI(-4-57) inhibitors were determined by titration of the activity of a known amount of active-site-titrated trypsin. For testing against kallikrein and trypsin, each KPI(-4-57) variant (0.5 to 100nM) was incubated with protease in low-binding 96-well microtiter plates at 30°C for 15-25 min, in 100mM Tris-HCl, pH 7.5, with 500mM NaCl, 5mM KCl, 5mM CaCl₂, 5mM MgCl₂, 0.1% Difco gelatin, and 0.05% Triton X-100. Chromogenic synthetic substrate was then be added, and initial rates at 30°C recorded by the SOFTmax kinetics program via a THERMOMax microplate reader (Molecular Devices Corp., Menlo Park, CA). The substrates used were N-α-benzoyl-L-Arg p-nitroanilide (1mM) for trypsin (20nM), and N-benzoyl-Pro-Phe-Arg p-nitroanilide (0.3mM) for plasma kallikrein (1nM). The Enzfitter (Elsevier) program was used both to plot fractional activity (i.e., activity with inhibitor, divided by activity without inhibitor), a, versus total concentration of inhibitor, I_t, and to calculate the dissociation constant of the inhibitor (K_i) by fitting the curve to the following equation:

$$a = 1 - \frac{[E]_t + [I]_t + K_i - \sqrt{([E]_t + [I]_t + K_i)^2 - 4[E]_t[I]_t}}{2[E]_t}$$

The K_s determined for purified KPI variants are shown in Figure 45. The most potent variants, KPI (-4-57; M15A, S17F) DD185 and KPI (-4-57; M15A, S17Y) TW6166 are 115-fold and 100-fold more potent,

respectively, as a human kallikrein inhibitor than wild-type KPI (-4-57). The least potent variant, KPI (-4-57; I16H, S17W) TW6185 is still 35-fold more potent than wild-type KPI.

5 For testing against factor XIIa, essentially the same reaction conditions were used, except that the substrate was N-benzoyl-Ile-Glu-Gly-Arg p-nitroaniline hydrochloride and its methyl ester (obtained from Pharmacia Hepar, Franklin, OH), and corn trypsin inhibitor (Enzyme Research Laboratories, South Bend, IN) was used as a control inhibitor. Factor XIIa was also obtained from Enzyme Research Laboratories.

10 Various data for inhibition of the serine proteases of interest kallikrein, plasmin, and factors Xa, XIa, and XIIa by a series of KPI variants are given in Figure 46. The results indicate that KPI variants can be produced that can bind to and preferably inhibit the activity of serine proteases. The results also indicate that the peptides of the invention may exhibit the preferable more potent and specific inhibition of one or more serine proteases of interest.

Example 5. Effect of KPI variant KPI185-1 on postoperative bleeding

25 A randomized, double-blinded study using an acute porcine cardiopulmonary bypass (CPB) model was used to investigate the effect of KPI185-1 on postoperative bleeding. Sixteen pigs (55-65 kg) underwent 60 minutes of hypothermic (28°C) open-chest CPB with 30 minutes of cardioplegic cardiac arrest. Pigs were randomized against a control solution of physiological saline (NS; n=8) or KPI-185 (n=8) groups. During aortic cross-clamping, the tricuspid valve was inspected through an atriotomy which was subsequently repaired. Following reversal of heparin with protamine, dilateral thoracostomy tubes were placed and shed blood collected for 3 hours. Shed blood volume and hemoglobin (Hgb) loss were calculated from total chest tube output and residual intrathoracic blood at time of sacrifice.

Total blood loss was significantly reduced in the KPI185-1 group (245.75 ± 66.24 ml vs. 344.25 ± 63.97 ml, $p=0.009$). In addition, there was a marked reduction in total Hgb loss in the treatment group (13.59 ± 4.26 gm vs. 23.61 ± 4.69 gm, $p=0.0005$). Thoracostomy drainage Hgb was significantly increased at 30 and 60 minutes in the control group [6.89 ± 1.44 vs. 4.41 ± 1.45 gm/dl ($p=0.004$) and 7.6 ± 1.03 vs. 5.26 ± 1.04 gm/dl ($p=0.0002$), respectively]. Preoperative and post-CPB hematocrits were not statistically different between the groups. These results are shown in graphical form in Figures 47-50.

The invention has been disclosed broadly and illustrated in reference to representative embodiments described above. Those skilled in the art will recognize that various modifications can be made to the present invention without departing from the spirit and scope thereof.

What Is Claimed Is:

1. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-X³-
 Cys-Arg-Ala-X⁴-X⁵-X⁶-X⁷-Trp-Tyr-Phe-Asp-
 Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X⁸-
 Tyr-Gly-Gly-Cys-X⁹-X¹⁰-X¹¹-X¹²-Asn-Asn-Phe-
 Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-
 Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X² is selected from Thr, Val, Ile and Ser;

X³ is selected from Pro and Ala;

X⁴ is selected from Arg, Ala, Leu, Gly, or Met;

X⁵ is selected from Ile, His, Leu, Lys, Ala, or Phe;

X⁶ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁷ is selected from Arg, His, or Ala;

X⁸ is selected from Phe, Val, Leu, or Gly;

X⁹ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr;

X¹⁰ is selected from Ala, Arg, or Gly;

X¹¹ is selected from Lys, Ala, or Asn;

X¹² is selected from Ser, Ala, or Arg;

provided that:

when X⁴ is Arg, X⁶ is Ile;

when X⁶ is Arg, X⁴ is Ala or Leu; when X⁹ is Tyr, X⁸ is Ala or X⁵ is His; and

either X⁵ is not Ile; or X⁶ is not Ser; or X⁹ is not Leu, Phe, Met, Tyr, or Asn; or X¹⁰ is not Gly; or X¹¹ is not Asn; or X¹² is not Arg.

2. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-
 Pro-Cys-Arg-Ala-X²-X³-X⁴-Arg-Trp-Tyr-Phe-
 Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-
 Phe-Tyr-Gly-Gly-Cys-X⁵-Gly-Asn-Arg-Asn-

Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-
Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X² is selected from Ala, Leu, Gly, or Met;

X³ is selected from Ile, His, Leu, Lys, Ala, or Phe;

X⁴ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁵ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr;

provided that:

when X¹ is Arg, X² is Ala or Leu; when X³ is Tyr, X⁴ is Ala or X⁵ is His; and

either X³ is not Ile; or X⁴ is not Ser; or X⁵ is not Leu, Phe, Met, Tyr, or Asn.

3. A protease inhibitor comprising the sequence:

Glu-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-
Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X¹-X²-X³-
Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-
Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X⁴-
Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-
Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Ala, Leu, Gly, or Met;

X² is selected from Ile, His, Leu, Lys, Ala, or Phe;

X³ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁴ is selected from Gly, Arg, Leu, Met, or Tyr;

provided that:

when X¹ is Ala, X² is Ile, His, or Leu;

when X¹ is Leu, X² is Ile or His;

when X¹ is Leu and X² is Ile, X³ is not Ser;

when X¹ is Gly, X² is Ile;

when X³ is Arg, X⁴ is Ala or Leu;

when X⁴ is Tyr, X⁵ is Ala or X⁵ is His; and

either X¹ is not Met, or X² is not Ile, or X³ is not Ser, or X⁴ is not Gly.

4. A protease inhibitor according to claim 1, wherein at least two amino acid residues selected from the group consisting of X⁴, X⁵, X⁶, and X⁷ differ from the residues found in the naturally occurring sequence of KPI.

5. A protease inhibitor according to claim 1, wherein X¹ is Asp or Glu, X² is Thr, X³ is Pro, and X¹² is Ser.

6. A protease inhibitor according to claim 5, wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, and X¹¹ is Asn.

7. A protease inhibitor according to claim 5, wherein X¹ is Asp, X² is Thr, X³ is Pro, X⁴ is Arg, X⁵ is Ile, X⁶ is Ile, X⁷ is Arg, X⁸ is Val, X⁹ is Arg, X¹⁰ is Ala, and X¹¹ is Lys.

8. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Ala.

9. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

10. A protease inhibitor according to claim 1, wherein X¹ is Glu, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Ala, X¹¹ is Asn, and X¹² is Arg.

11. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pr, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Arg, X¹¹ is Asn, and X¹² is Arg.

12. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Arg, X⁸ is Val, Leu, or Gly, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg.

13. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Met, X⁵ is Ile, X⁶ is Ser, X⁷ is Ala, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Asn, and X¹² is Arg.

14. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Pro, X⁴ is Ala or Leu, X⁵ is Ile, X⁶ is Tyr, X⁷ His, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

15. A protease inhibitor according to claim 14, wherein X³ is Thr, and X⁴ is Ala.

16. A protease inhibitor according to claim 14, wherein X³ is Thr, and X⁴ is Leu.

17. A protease inhibitor according to claim 14, wherein X³ is Val, and X⁴ is Ala.

18. A protease inhibitor according to claim 14, wherein X³ is Ser, and X⁴ is Ala.

19. A protease inhibitor according to claim 14, wherein X³ is Val, and X⁴ is Leu.

20. A protease inhibitor according to claim 14, wherein X³ is Ser, and X⁴ is Leu.

21. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Gly, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

22. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Tyr, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

23. A protease inhibitor according to claim 1, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Pro, X⁴ is Leu, X⁵ is Phe, X⁶ is Lys, X⁷ is Arg, X⁸ is Phe, X⁹ is Leu, X¹⁰ is Gly, X¹¹ is Ala, and X¹² is Arg.

24. A protease inhibitor according to claim 2, wherein X¹ is Glu, X² is Met, X³ is Ile, X⁴ is Ile, and X⁵ is Gly.

25. A protease inhibitor according to claim 3, wherein X¹ is Met, X² is Ser, and X³ is Gly.

26. A protease inhibitor according to claim 25, wherein X² is selected from His, Ala, Phe, Lys, and Leu.

27. A protease inhibitor according to claim 26, wherein X² is His.

28. A protease inhibitor according to claim 27, wherein X² is Ala.

29. A protease inhibitor according to claim 27, wherein X² is Phe.

30. A protease inhibitor according to claim 27, wherein X² is Lys.

31. A protease inhibitor according to claim 27, wherein X² is Leu.

32. A protease inhibitor according to claim 3, wherein X¹ is Met, X² is Ile, and X³ is Gly.

33. A protease inhibitor according to claim 32, wherein X³ is Ile.

34. A protease inhibitor according to claim 32, wherein X³ is Pro.

35. A protease inhibitor according to claim 32, wherein X³ is Phe.

36. A protease inhibitor according to claim 32, wherein X³ is Tyr.

37. A protease inhibitor according to claim 32, wherein X³ is Trp.

38. A protease inhibitor according to claim 32, wherein X³ is Asn.

39. A protease inhibitor according to claim 32, wherein X³ is Leu.

40. A protease inhibitor according to claim 32, wherein X³ is Lys.

41. A protease inhibitor according to claim 32, wherein X³ is His.

42. A protease inhibitor according to claim 32, wherein X³ is Glu.

43. A protease inhibitor according to claim 3, wherein X¹ is Ala.

44. A protease inhibitor according to claim 43, wherein X² is Ile.

45. A protease inhibitor according to claim 44, wherein X³ is Phe, and X⁴ is Gly.

46. A protease inhibitor according to claim 44, wherein X³ is Tyr, and X⁴ is Gly.

47. A protease inhibitor according to claim 44, wherein X³ is Trp, and X⁴ is Gly.

48. A protease inhibitor according to claim 44, wherein X³ is Ser or Phe, and X⁴ is Arg or Tyr.

49. A protease inhibitor according to claim 43, wherein X² is His or Leu, X³ is Phe, and X⁴ is Gly.

50. A protease inhibitor according to claim 3, wherein X¹ is Leu.

51. A protease inhibitor according to claim 50, wherein X² is His, X³ is Asn or Phe, and X⁴ is Gly.

52. A protease inhibitor according to claim 50, wherein X² is Ile, X³ is Pro, and X⁴ is Gly.

53. A protease inhibitor according to claim 3, wherein X¹ is Gly, X² is Ile, X³ is Tyr, and X⁴ is Gly.

54. A protease inhibitor according to claim 3, wherein X¹ is Met, X² is His, X³ is Ser, and X⁴ is Tyr.

55. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 1.

56. An isolated DNA molecule according to claim 55, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

57. An isolated DNA molecule according to claim 56, further comprising a DNA sequence encoding a secretory signal peptide.

58. An isolated DNA molecule according to claim 57, wherein said secretory signal peptide comprises the signal sequence of yeast alpha-mating factor.

59. A host cell transformed with a DNA molecule according to claim 55.

60. A host cell according to claim 59, wherein said host cell is *E. coli* or a yeast cell.

61. A host cell according to claim 60, wherein said yeast cell is selected from *Pichia pastoris* and *Saccharomyces cerevisiae*.

62. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 59 and isolating and purifying said protease inhibitor.

63. A pharmaceutical composition, comprising a protease inhibitor according to claim 1, together with a pharmaceutically acceptable sterile vehicle.

64. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 63.

65. The method of treatment of claim 64, wherein said clinical condition is blood loss during surgery.

66. A method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition according to claim 63.

67. The method of claim 66, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

68. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-X²-Gly-Pro-
Cys-Arg-Ala-X³-X⁴-X⁵-X⁶-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-
Tyr-Gly-Gly-Cys-X⁷-Gly-Asn-Arg-Asn-Asn-
Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-
Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X² is selected from Thr, Val, Ile and Ser;

X³ is selected from Arg, Ala, Leu, Gly, or Met;

X⁴ is selected from Ile, His, Leu, Lys, Ala, or Phe;

X⁵ is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X⁶ is selected from Arg, His, or Ala; and

X⁷ is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr.

69. A protease inhibitor according to claim 68, wherein at least two amino acid residues selected from the group consisting of X³, X⁴, X⁵, and X⁶ differ from the residues found in the naturally occurring sequence of KPI.

70. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, Val, or Ser, X³ is Ala or Leu, X⁴ is Ile, X⁵ is Tyr, X⁶ is His and X⁷ is Gly.

71. A protease inhibitor according to claim 70, wherein X¹ is Thr, and X² is Ala.

72. A protease inhibitor according to claim 70, wherein X¹ is Thr, and X² is Leu.

73. A protease inhibitor according to claim 70, wherein X² is Val, and X³ is Ala.

74. A protease inhibitor according to claim 70, wherein X² is Ser, and X³ is Ala.

75. A protease inhibitor according to claim 70, wherein X² is Val, and X³ is Leu.

76. A protease inhibitor according to claim 70, wherein X² is Ser, and X³ is Leu.

77. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Gly.

78. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Tyr.

79. A protease inhibitor according to claim 68, wherein X¹ is Glu-Val-Val-Arg-Glu-, X² is Thr, X³ is Leu, X⁴ is Phe, X⁵ is Lys, X⁶ is Arg and X⁷ is Leu.

80. A protease inhibitor comprising the sequence:

X¹-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-
Pro-Cys-X²-Ala-X³-X⁴-X⁵-X⁶-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-
Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-
Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-
Cys-Gly-Ser-Ala-Ile,

wherein:

X¹ is selected from Glu-Val-Val-Arg-Glu- and Asp-Val-Val-Arg-Glu-;

X² is selected from Arg and Lys;

X³ is selected from Met, Arg, Ala, Leu, Ser, Val;

X⁴ is selected from Ile and Ala;

X⁵ is selected from Ser, Ile, Ala, Pro, Phe, Tyr, and Trp; and

X⁶ is selected from Arg, Ala, His, Gln, and Thr;
provided that:

when X¹ is Arg, X² is Leu, and X⁴ is Ile, X⁵ cannot be Ser; and also provided that either X³ is not Met; or X⁴ is not Ile; or X⁵ is not Ser; or X⁶ is not Arg.

81. A protease inhibitor according to claim 80, wherein X⁵ is selected from Phe, Tyr and Trp.

82. A protease inhibitor according to claim 80, wherein X⁴ is Ile.

83. A protease inhibitor according to claim 82, wherein X² is Lys.

84. A protease inhibitor according to claim 83, wherein X³ is Met.

85. A protease inhibitor according to claim 84, wherein X⁵ is Ser.

86. A protease inhibitor according to claim 84, wherein X⁵ is Ile.

87. A protease inhibitor according to claim 83, wherein X³ is Arg.

88. A protease inhibitor according to claim 87, wherein X⁵ is Ser.

89. A protease inhibitor according to claim 87, wherein X⁵ is Ile.

90. A protease inhibitor according to claim 82, wherein X¹ is Arg.

91. A protease inhibitor according to claim 90, wherein X¹ is Arg or Met, and X² is Ser or Ile.

92. A protease inhibitor according to claim 91, wherein X¹ is Arg.

93. A protease inhibitor according to claim 92, wherein X¹ is Ser.

94. A protease inhibitor according to claim 92, wherein X¹ is Ile.

95. A protease inhibitor according to claim 91, wherein X¹ is Met.

96. A protease inhibitor according to claim 95, wherein X¹ is Ser.

97. A protease inhibitor according to claim 95, wherein X¹ is Ile.

98. A protease inhibitor according to claim 82, wherein X¹ is Ala.

99. A protease inhibitor according to claim 82, wherein X¹ is Leu.

103. A protease inhibitor according to claim 82, wherein X³ is Phe.

104. A protease inhibitor according to claim 82, wherein X³ is Tyr.

105. A protease inhibitor according to claim 82, wherein X³ is Trp.

106. A protease inhibitor according to claim 104, wherein X³ is Ala or Leu.

107. A protease inhibitor according to claim 106, wherein X³ is Ala.

108. A protease inhibitor according to claim 106, wherein X³ is Leu.

109. A protease inhibitor according to claim 105, wherein X³ is Ala.

110. A protease inhibitor according to claim 109, wherein X³ is His.

111. A protease inhibitor according to claim 109, wherein X³ is Gln.

112. A protease inhibitor according to claim 109, wherein X³ is Thr.

113. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 80.

114. An isolated DNA molecule according to claim 113, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

115. An isolated DNA molecule according to claim 114, further comprising a DNA sequence encoding a secretory signal peptide.

116. An isolated DNA molecule according to claim 115, wherein said secretory signal peptide comprises the signal sequence of yeast alpha-mating factor.

117. A host cell transformed with a DNA molecule according to claim 113.

118. A host cell according to claim 117, wherein said host cell is *E. coli* or a yeast cell.

119. A host cell according to claim 118, wherein said yeast cell is selected from *Pichia pastoris* and *Saccharomyces cerevisiae*.

120. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 117 and isolating and purifying said protease inhibitor.

121. A pharmaceutical composition, comprising a protease inhibitor according to claim 80, together with a pharmaceutically acceptable sterile vehicle.

122. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 121.

123. The method of treatment of claim 122, wherein said clinical condition is blood loss during surgery.

124. A method for inhibiting the activity of serine proteases of interest in a mammal comprising

administering a therapeutically effective dose of a pharmaceutical composition according to claim 121.

125. The method of claim 124, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

126. A protease inhibitor according to claim 81, wherein X⁴ is Ile.

127. A protease inhibitor according to claim 126, wherein X⁵ is Phe.

128. A protease inhibitor according to claim 126, wherein X⁵ is Tyr.

129. A protease inhibitor according to claim 126, wherein X⁵ is Trp.

130. A protease inhibitor according to claim 128, wherein X⁵ is Ala or Leu.

131. A protease inhibitor according to claim 130, wherein X⁵ is Ala.

132. A protease inhibitor according to claim 130, wherein X⁵ is Leu.

133. A protease inhibitor according to claim 129, wherein X⁵ is Ala.

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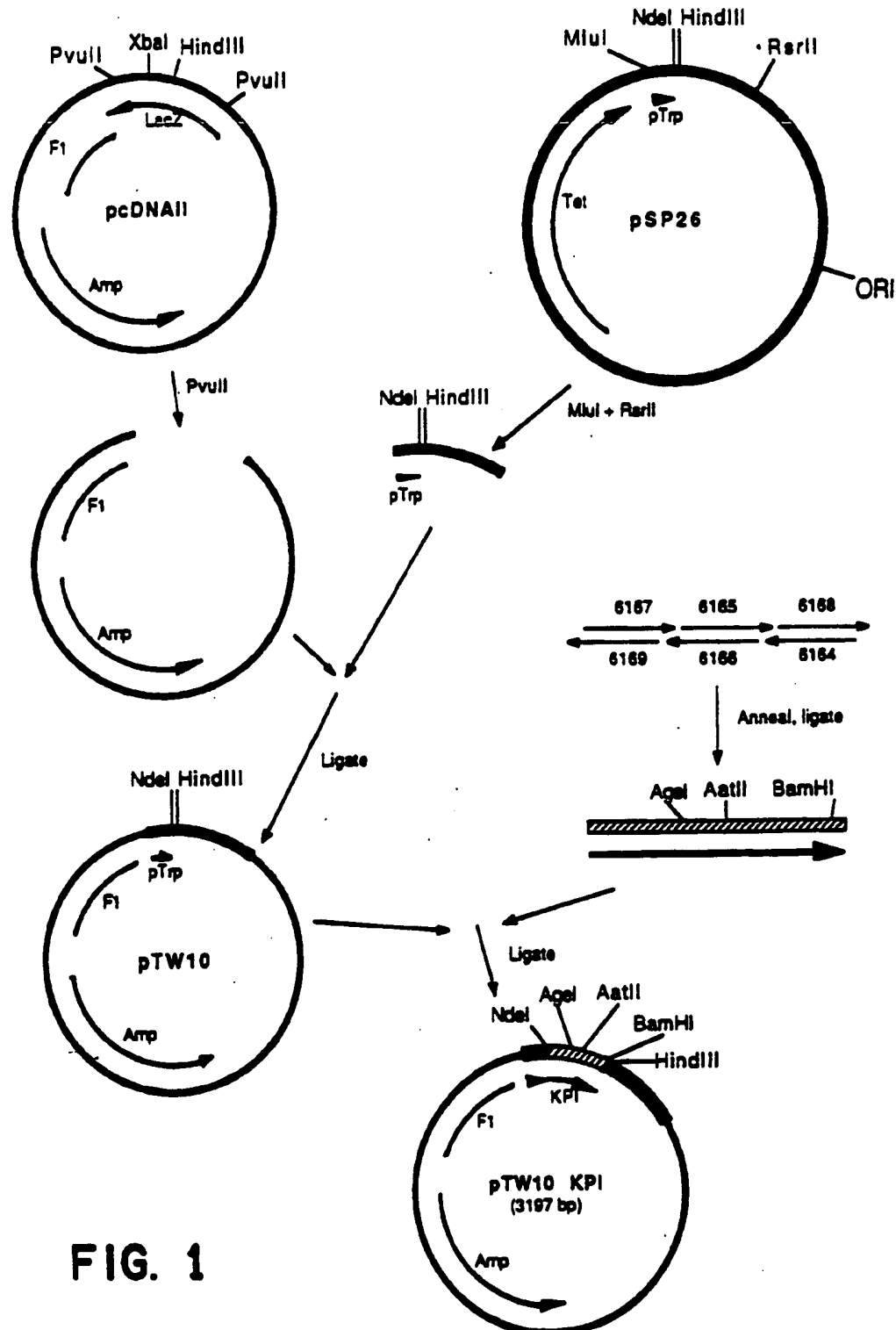


FIG. 1

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FIG. 2

NdeI
 TATG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC OCT CTG ACA AAA
 AC TTT GTT TCG TGA TAA CGT GAC CGT GAG AAT GGC AAT GAC AAA TGG CGA CAC TGT TTT
 ▶ Met Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys
 KPI
 GCG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
 CCG CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC
 ▶ Ala Glu Val Cys Ser Glu Glu Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp
 AgeI
 TAC TTT GAC GTC ACT GAA GGT AAG TGC TGC OCT CCA TTC TTT TAC GGC GGT TCC GGC GGC AAC
 ATG AAA CTG CAG TGA CTT CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG
 ▶ Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Cys Gly Gly Asn
 AatII
 CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TCC GGA TCC GCT ATT TA
 GCA TTG TTG AAA CTG TGA CTT CTC ATG ACG TAC CGT CAC ACG OCT AGG CGA TAA ATT CGA
 ▶ Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
 BamHI
 HindIII

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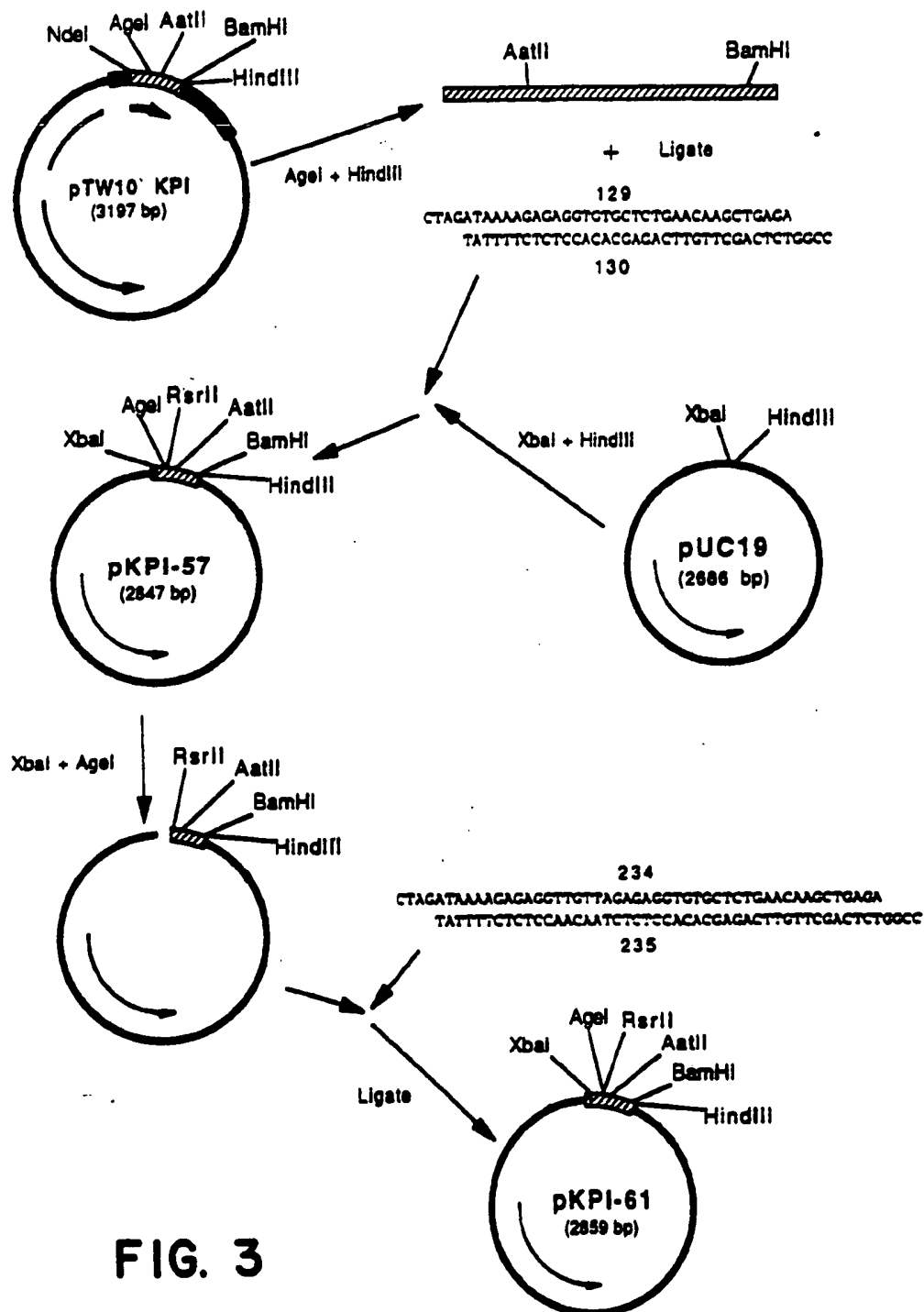


FIG. 3

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FIG. 4

XbaI				KPI (1-57)												RsrII			
																AgeI			
CTA	GAT	AAA	AGA	GAG	GTG	TGC	TCT	GAA	CAA	GCT	GAG	ACC	GGT	CCG	TGC	CGT			
	TA	TTT	TCT	CTC	CAC	ACG	AGA	CTT	GTT	CGA	CTC	TGG	CCA	GGC	ACG	OCA			
► Leu	Asp	Lys	Arg	Glu	Val	Cys	Ser	Glu	Gln	Ala	Glu	Thr	Gly	Pro	Cys	Arg			
												AatII							
GCA	ATG	ATC	TCC	CGC	TGG	TAC	TTT	GAC	GTC	ACT	GAA	GGT	AAG	TGC	GCT	CCA			
CGT	TAC	TAG	AGG	GCG	ACC	ATG	AAA	CTG	CAG	TGA	CTT	CCA	TTC	ACG	CGA	GGT			
► Ala	Met	Ile	Ser	Arg	Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys	Ala	Pro			
TTC	TTT	TAC	GGC	GGT	TGC	GGC	GGC	AAC	CGT	AAC	AAC	TTT	GAC	ACT	GAA	GAG			
AAG	AAA	ATG	CCG	CCA	ACG	CCG	CCG	TTG	GCA	TTG	TTG	AAA	CTG	TGA	CTT	CTC			
► Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	Arg	Asn	Asn	Phe	Asp	Thr	Glu	Glu			
												BamHI				HindIII			
TAC	TGC	ATG	GCA	GTG	TGC	GGA	TCC	GCT	ATT	TA									
ATG	ACG	TAC	CGT	CAC	ACG	CCT	AGG	CGA	TAA	ATT	CGA								
► Tyr	Cys	Met	Ala	Val	Cys	Gly	Ser	Ala	Ile										

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FIG. 5

RsrII

AgeI

KPI (-4-57)

XbaI

CTA	GAT	AAA	AGA	GAG	GTT	GTT	AGA	GAG	GTG	TGC	TCT	GAA	CAA	GCT	GAG	ACC	GGT
	TA	TTT	TCT	CTC	CAA	CAA	TCT	CTC	CAC	ACG	AGA	CTT	GTT	CGA	CTC	TGG	CCA
▶ Leu	Asp	Lys	Arg	Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	Ala	Glu	Thr	Gly

AatII

CCG	TGC	CGT	GCA	ATG	ATC	TCC	CGC	TGG	TAC	TTT	GAC	GTC	ACT	GAA	GGT	AAG	TGC
GGC	ACG	GCA	CGT	TAC	TAG	AGG	GCG	ACC	ATG	AAA	CTG	CAG	TGA	CTT	CCA	TTC	ACG
▶ Pro	Cys	Arg	Ala	Met	Ile	Ser	Arg	Trp	Tyr	Phe	Asp	Val	Thr	Glu	Gly	Lys	Cys

GCT	CCA	TTC	TTT	TAC	GGC	GGT	TGC	GGC	GGC	AAC	CGT	AAC	AAC	TTT	GAC	ACT	GAA
CGA	GGT	AAG	AAA	ATG	CCG	CCA	ACG	CCG	CCG	TTG	GCA	TTG	TTG	AAA	CTG	TGA	CTT
▶ Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	Arg	Asn	Asn	Phe	Asp	Thr	Glu

BamHI

HindIII

GAG	TAC	TGC	ATG	GCA	GTG	TGC	GGA	TCC	GCT	ATT	TA	
CTC	ATG	ACG	TAC	CGT	CAC	ACG	CCT	AGG	CGA	TAA	ATT	CGA
▶ Glu	Tyr	Cys	Met	Ala	Val	Cys	Gly	Ser	Ala	Ile		

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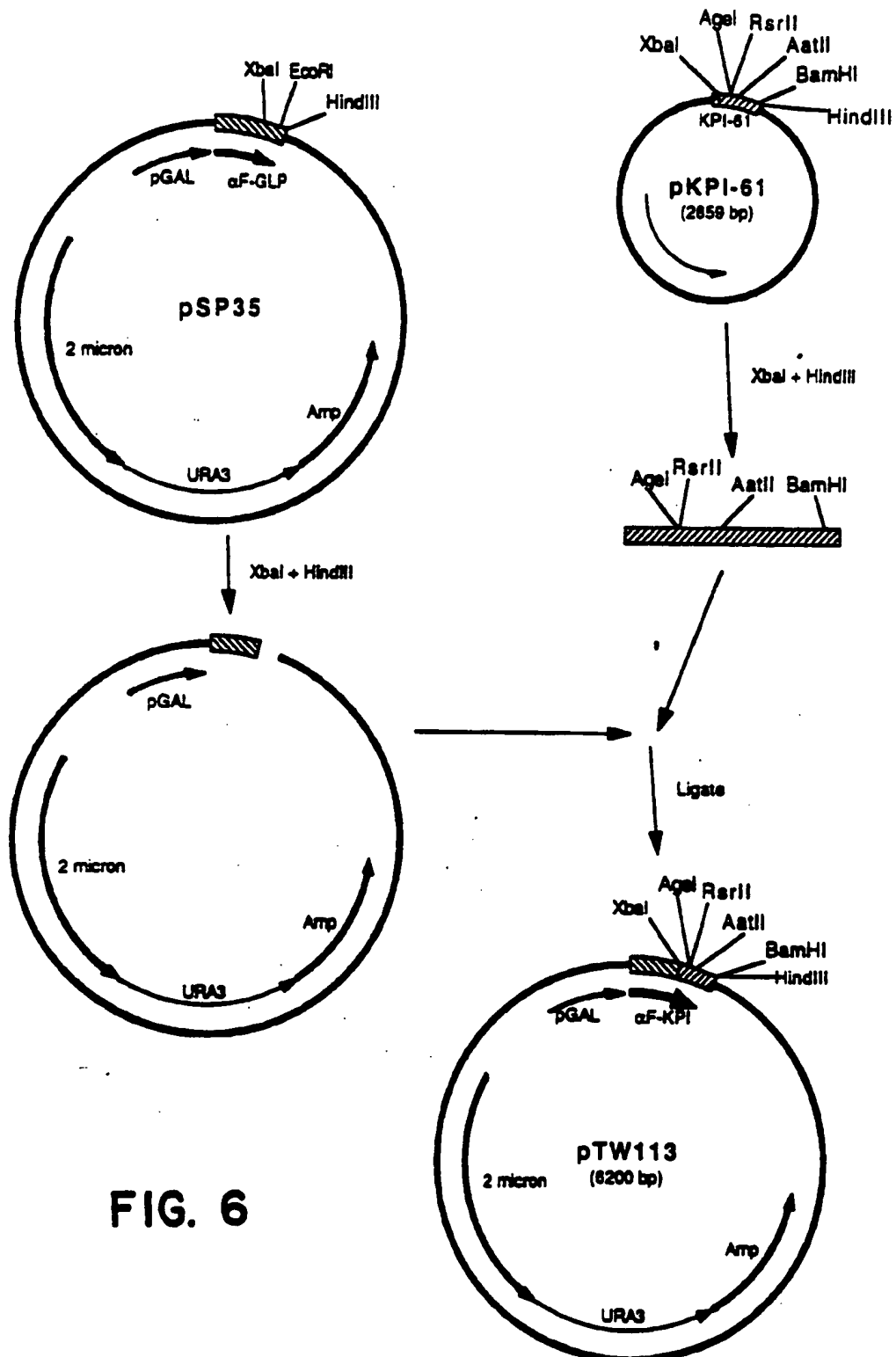


FIG. 6

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FIG. 7

α-factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CCG TCG TAA CGA CGA TTT
▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI **KPI(-4-57)**

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII **AgeI** **AatII**

GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC ATG AAA CTG CAG TGA CTT
▶ Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu

GST AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI **HindIII**

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

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FIG. 8

KPI(-4-57)

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - Ile - Ser - Arg
8 9 10 11 12 13 14 15 16 17 18

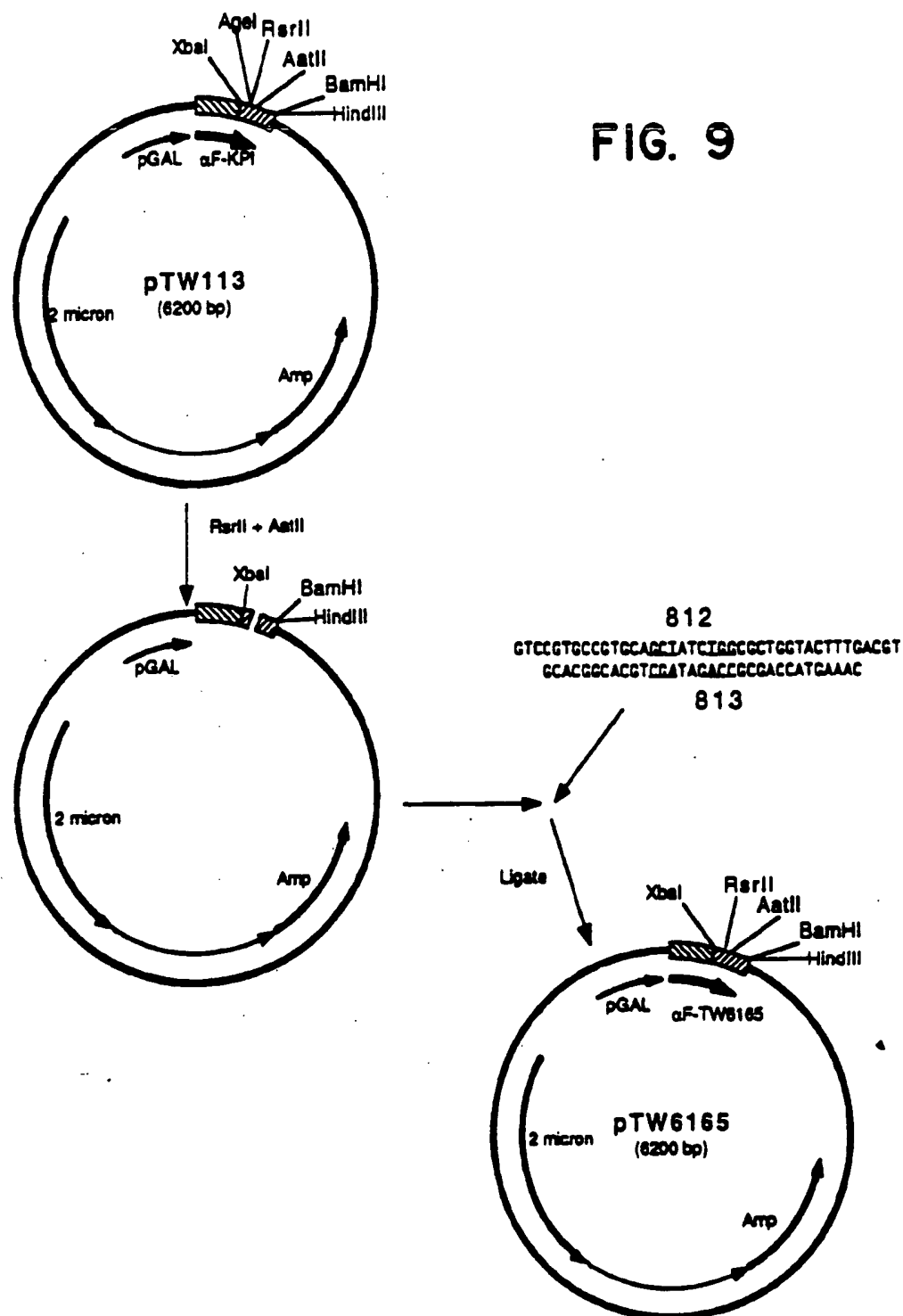
Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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pTW 6165

FIG. 10

 α -factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
 ▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

 GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
 ▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

 ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
 ▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

 AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CCG TCG TAA CGA CGA TTT
 ▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI

KPI(-4-57; M15A, S17W)

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
 ▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI

AatII

GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TGG GGC TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ACC GCG ACC ATG AAA CTG CAG TGA CTT
 ▶ Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Trp Arg Trp Tyr Phe Asp Val Thr Glu

 GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
 ▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI

HindIII

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT ACG CGA TAA ATT CGA A
 ▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

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FIG. II

812 GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT GCACGGCACGTCCATAGACCGGACCATGAAAC 813	pTW6165 KPI(-4-57; M15A, S17F)
814 GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT GCACGGCACGTCCATAGATGGCGACCATGAAAC 815	pTW6166 KPI(-4-57; M15A, S17Y)
867 GTCCGTGCCGTGCATTCATCTTCGGCTGGTACTTTGACGT GCACGGCACGTAACTAGAAAGCGACCATGAAAC 868	pTW6175 KPI(-4-57; M15L, S17F)
1493 GTCCGTGCCGTGCATTCATCTACCGCTGGTACTTTGACGT GCACGGCACGTAACTAGATGGCGACCATGAAAC 1494	pBG028 KPI(-4-57; M15L, S17Y)
925 GTCCGTGCCGTGCAATGCACTTCGGCTGGTACTTTGACGT GCACGGCACGTTACGTGAAGCGGACCATGAAAC 926	pTW6183 KPI(-4-57; I16H, S17F)
927 GTCCGTGCCGTGCAATGCACTACCGCTGGTACTTTGACGT GCACGGCACGTTACGTGATGGCGACCATGAAAC 928	pTW6184 KPI(-4-57; I16H, S17Y)
929 GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT GCACGGCACGTTACGTGACCGCGACCATGAAAC 930	pTW6185 KPI(-4-57; I16H, S17W)
863 GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT GCACGGCACGTCTAGTCAGGCGGACCATGAAAC 864	pTW6173 KPI(-4-57; M15A, I16H)
865 GTCCGTGCCGTGCATTTCACCTCCCGCTGGTACTTTGACGT GCACGGCACGTAACTGAGGCGGACCATGAAAC 866	pTW6174 KPI(-4-57; M15L, I16H)

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pTW 6166

FIG. 12

α-factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI **KPI(-4-57; M15A, S17Y)**

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI **AatII**

GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
▶ Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

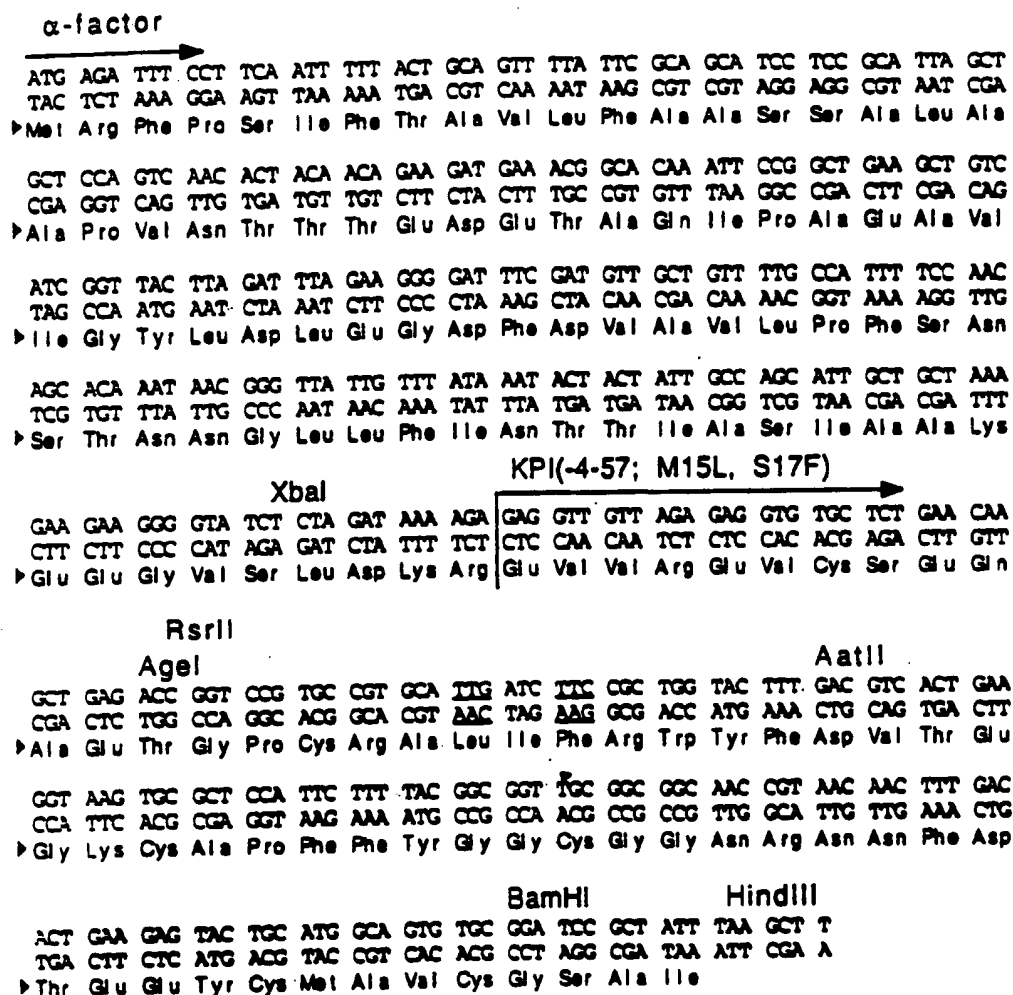
GCT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI **HindIII**

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

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FIG. 13



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FIG. 14

 α -factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
 ▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
 ▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
 ▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TAA CCG TCG TAA CGA CGA TTT
 ▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI

KPI(-4-57; M15L, S17Y)

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
 ▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI

AatII

GCT GAG ACC GGT CCG TGC CGT GCA TTG ATC TAC CCG TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT AAC TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
 ▶ Ala Glu Thr Gly Pro Cys Arg Ala Leu Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
 ▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

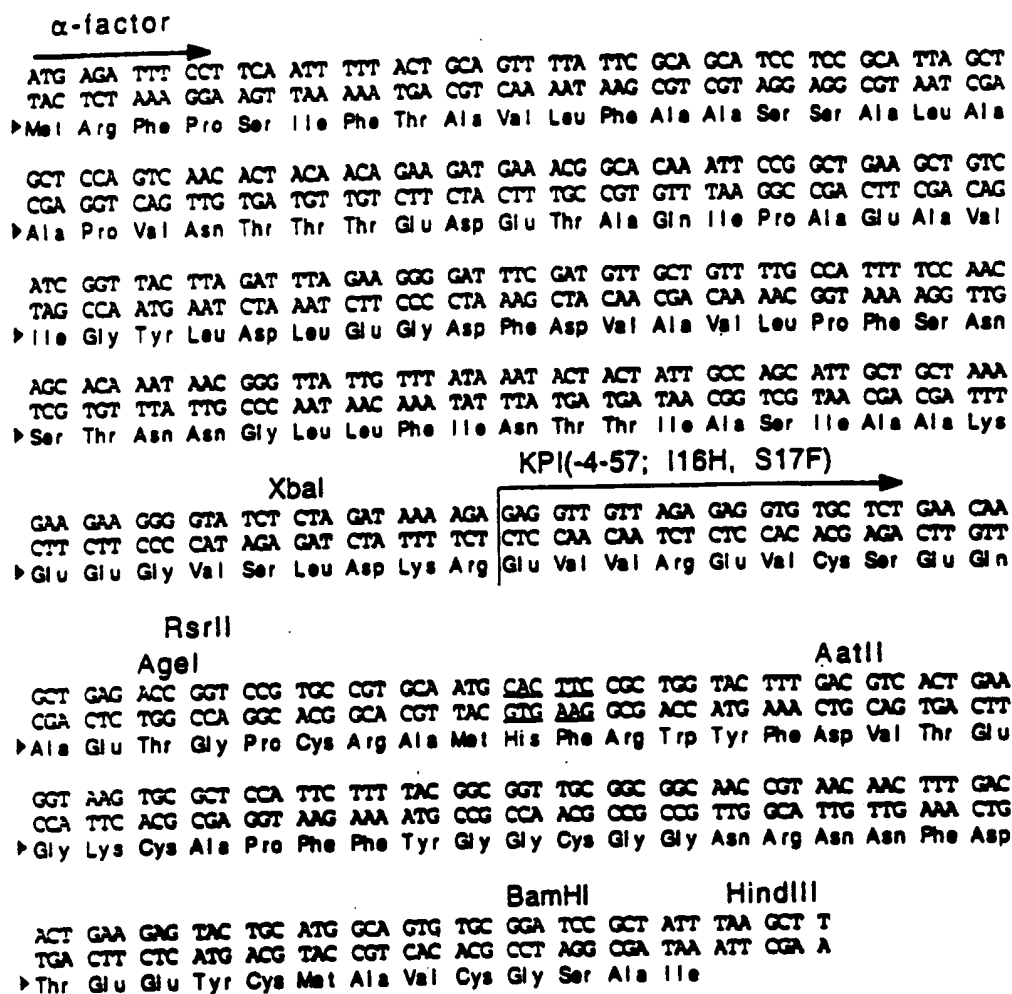
BamHI

HindIII

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
 ▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

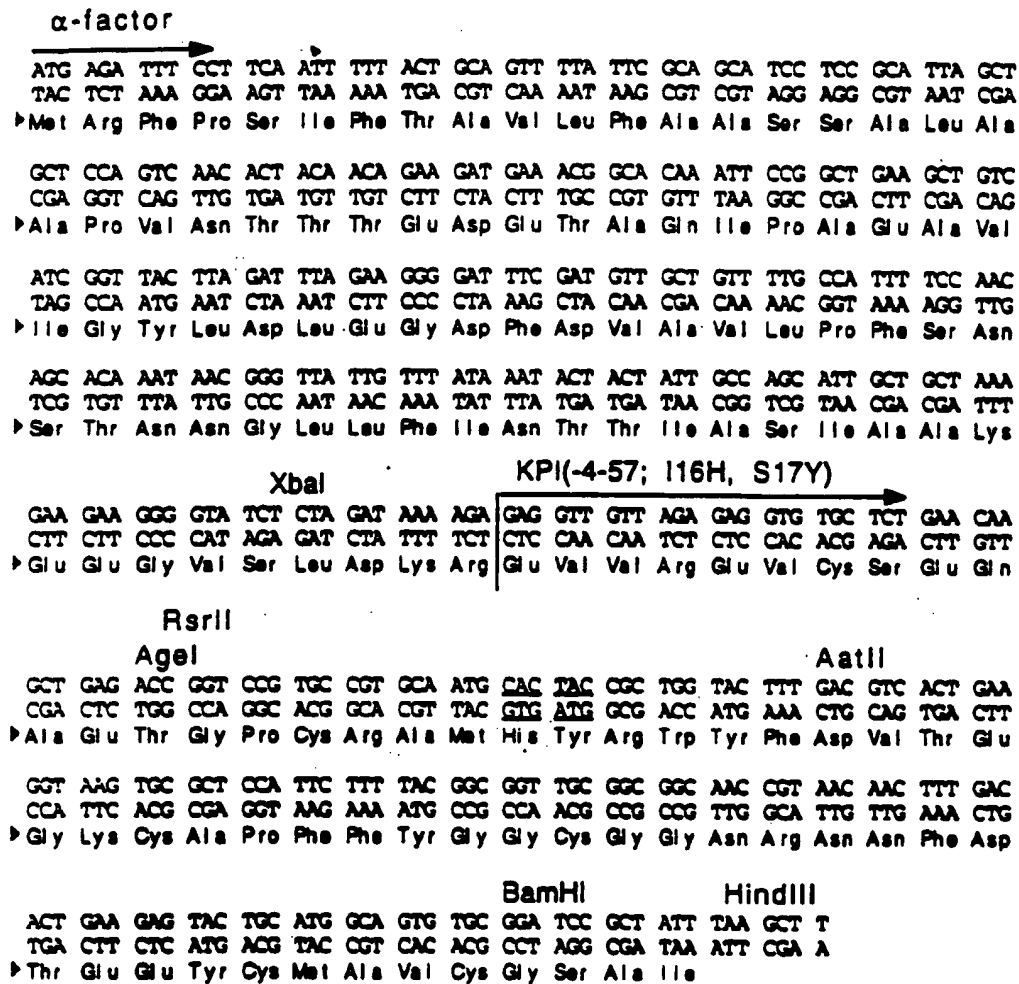
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FIG. 15



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FIG. 16



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FIG. 17

α -factor
 ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
 ▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

 GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
 ▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

 ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
 ▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

 AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
 ▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

 XbaI
 GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
 ▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

 KPI(-4-57; I16H, S17W)

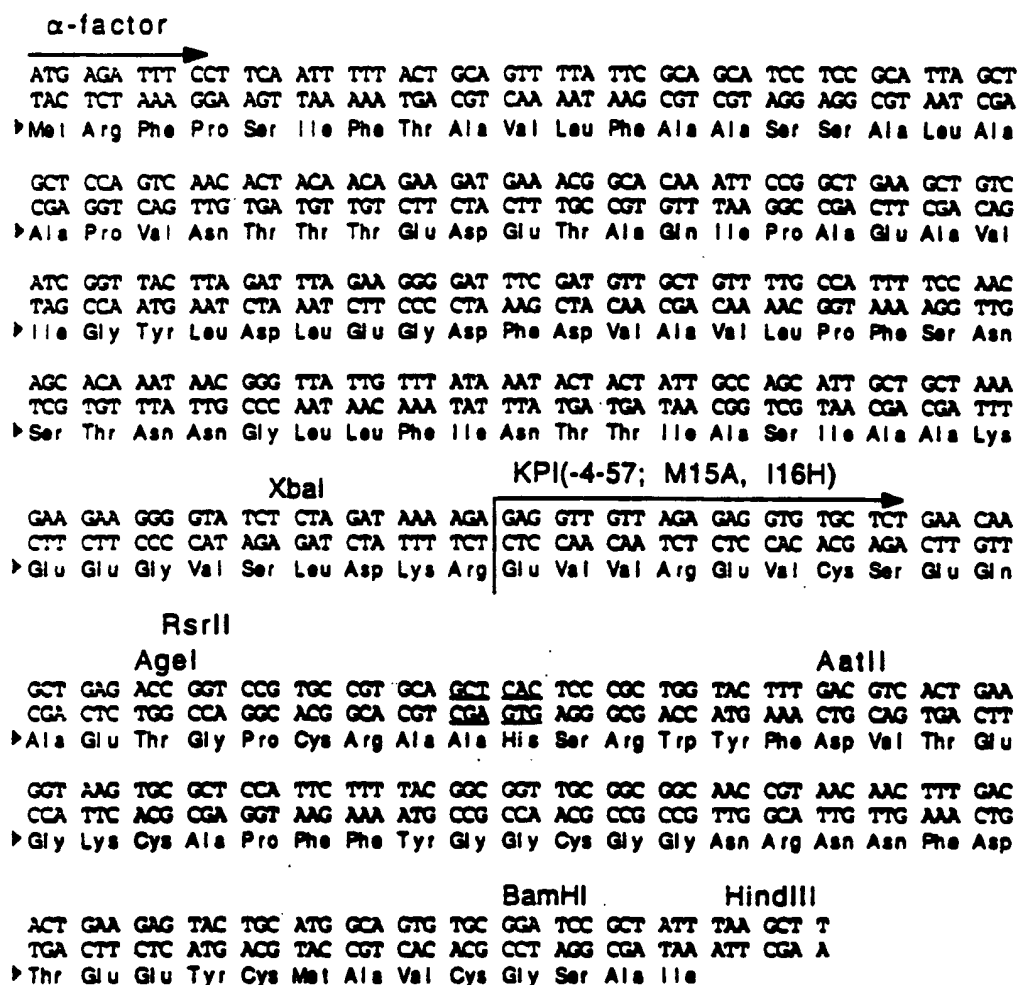
 RsrII
 AgeI
 GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TGG CGC TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG ACC GCG ACC ATG AAA CTG CAG TGA CTT
 ▶ Ala Glu Thr Gly Pro Cys Arg Ala Met His Trp Arg Trp Tyr Phe Asp Val Thr Glu

 AatII
 GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
 ▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

 BamHI
 HindIII
 ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
 ▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

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FIG. 18



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FIG. 19

α-factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▶ Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▶ Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▶ Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CCG TCG TAA CGA CGA TTT
▶ Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

KPI(-4-57; M15L, I16H)

XbaI

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▶ Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII

AgeI

GCT GAG ACC GGT CCG TGC CGT GCA TTG CAC TCC CCG TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC GTG AGG GCG ACC ATG AAA CTG CAG TGA CTT
▶ Ala Glu Thr Gly Pro Cys Arg Ala Leu His Ser Arg Trp Tyr Phe Asp Val Thr Glu

AatII

GCT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▶ Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▶ Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

HindIII

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FIG. 20

KPI(-4-57; M15A, S17W) TW6165

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
 -4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - ~~Ala~~ - Ile - ~~Trp~~ - Arg
 8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
 19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
 30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
 41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
 52 53 54 55 56 57

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FIG. 21

KPI(-4-57; M15A, S17Y) TW6166

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - ~~Ala~~ - Ile - ~~Tyr~~ - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 22

KPI(-4-57; M15L, S17F) TW6175

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Leu - Ile - Phe - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 23

KPI(-4-57; M15L, S17Y) BG028

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - ~~Leu~~ - ~~Ile~~ - ~~Tyr~~ - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 24

KPI(-4-57; I16H, S17F) TW6183

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - His - Phe - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 25

KPI(-4-57; I16H, S17Y) TW6184

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - His - Tyr - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 26

KPI(-4-57; I16H, S17W) TW6185

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - His - Icp - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 27

KPI(-4-57; M15A, S17F) DD185

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - ~~Ala~~ - Ile - ~~Phe~~ - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 28

KPI(-4-57; M15A, I16H) TW6173

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Ala - His - Ser - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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FIG. 29

KPI(-4-57; M15L, I16H) TW6174

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - ~~Leu~~ - ~~His~~ - Ser - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

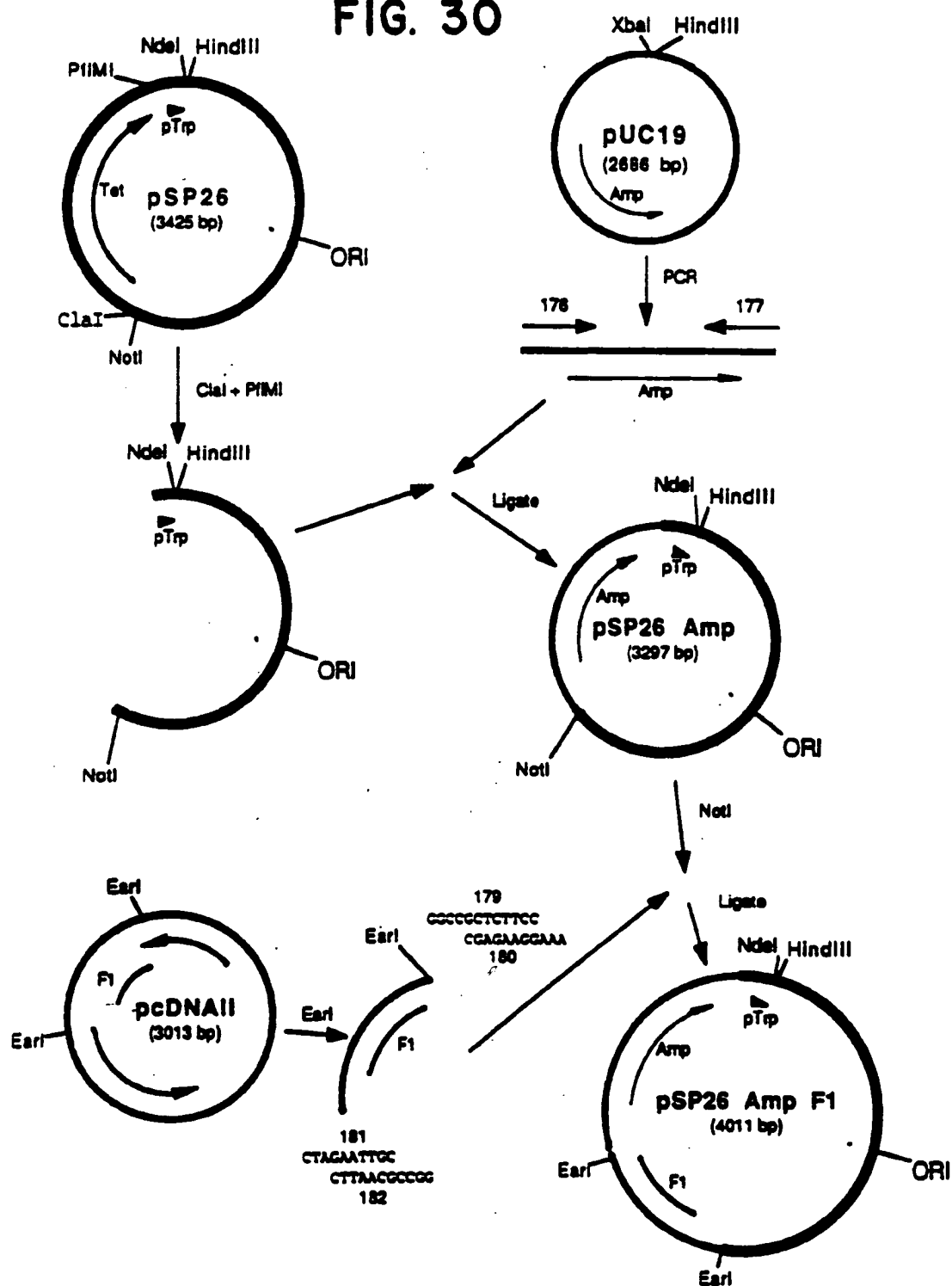
Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

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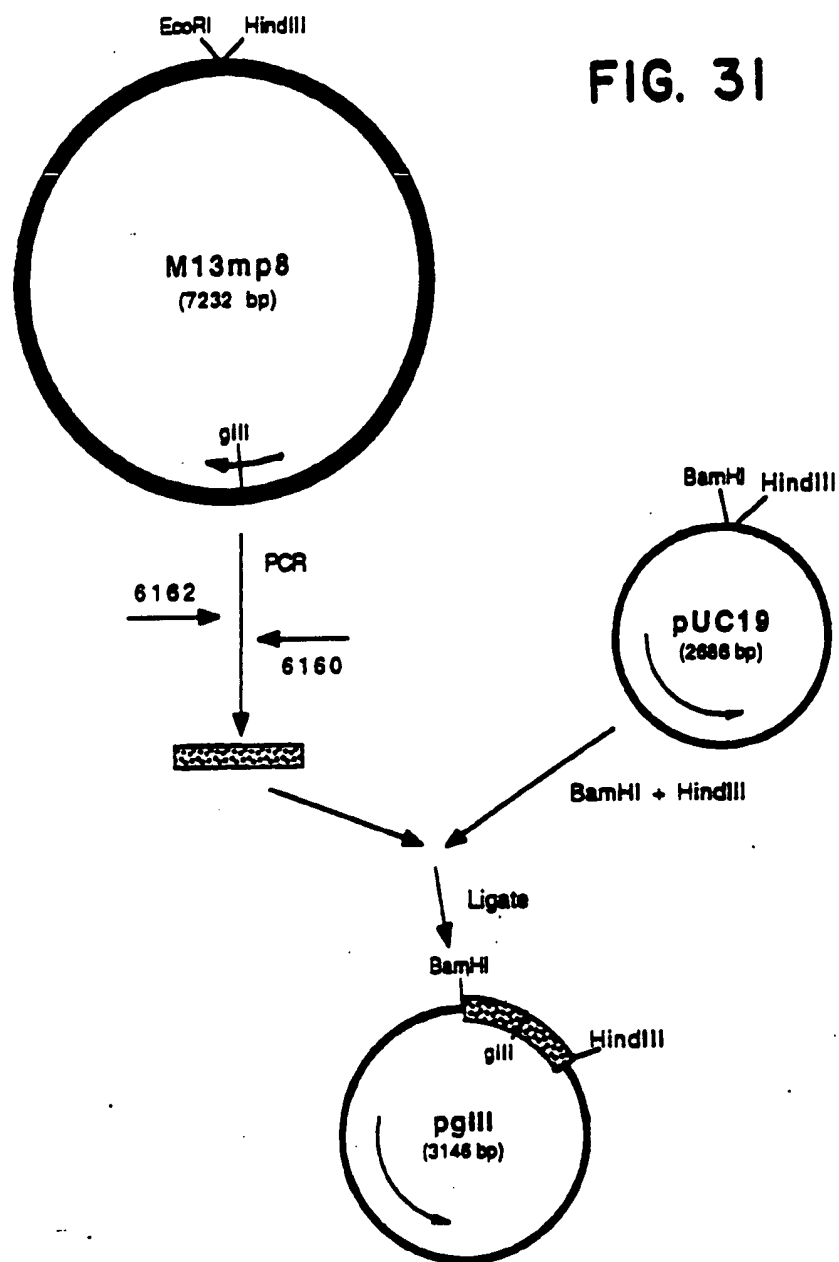
FIG. 30



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FIG. 31



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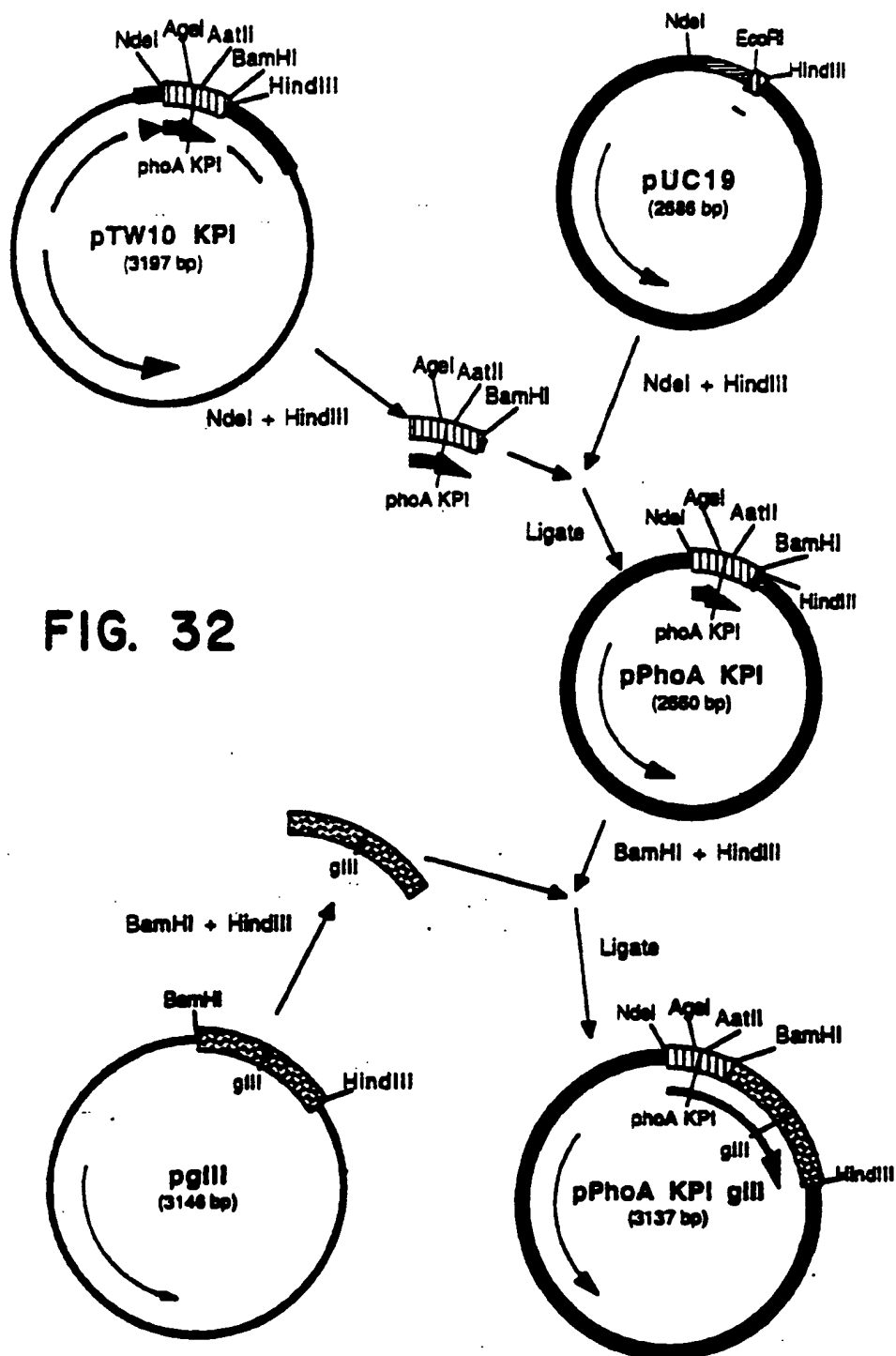
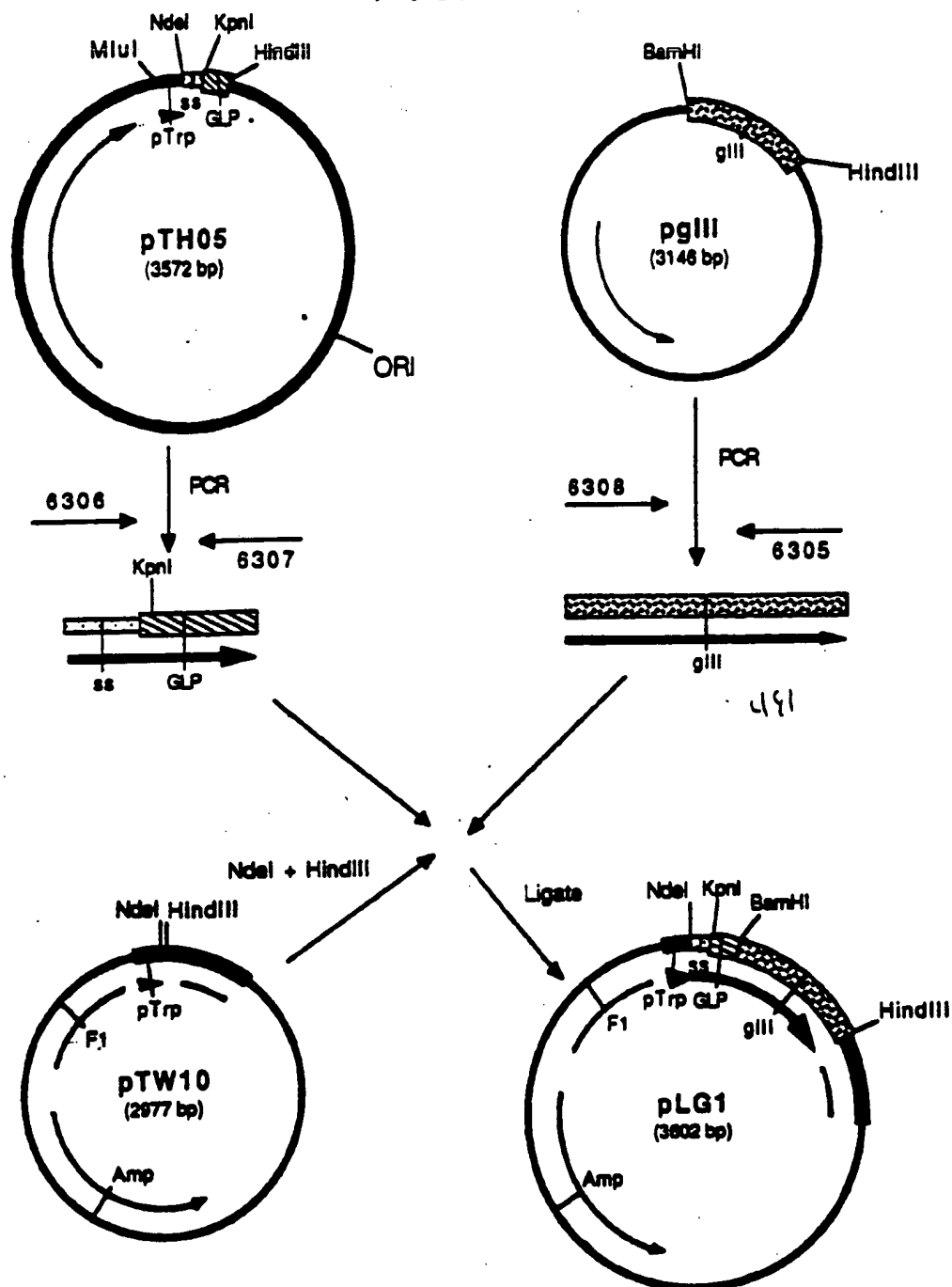


FIG. 32

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FIG. 33



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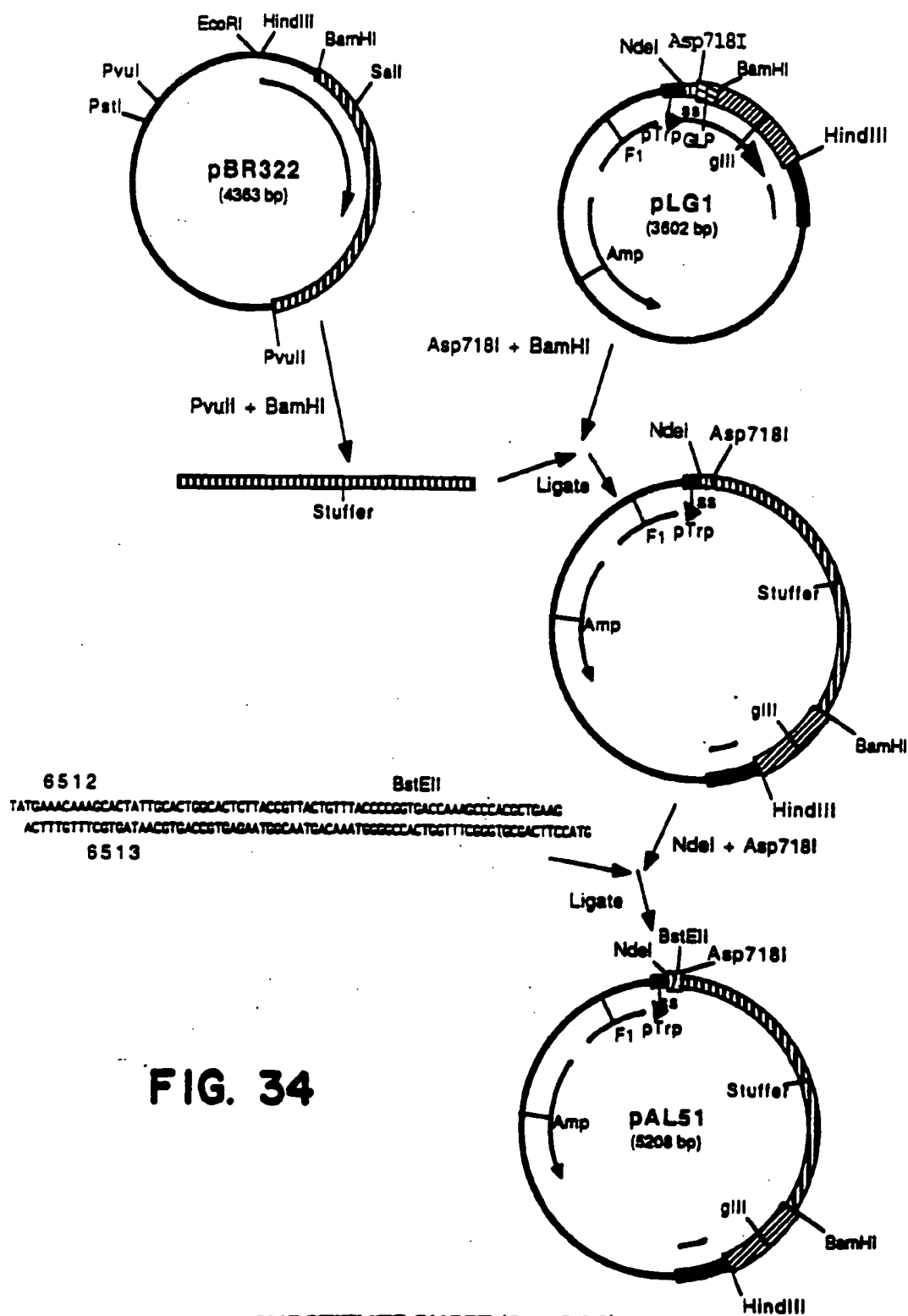
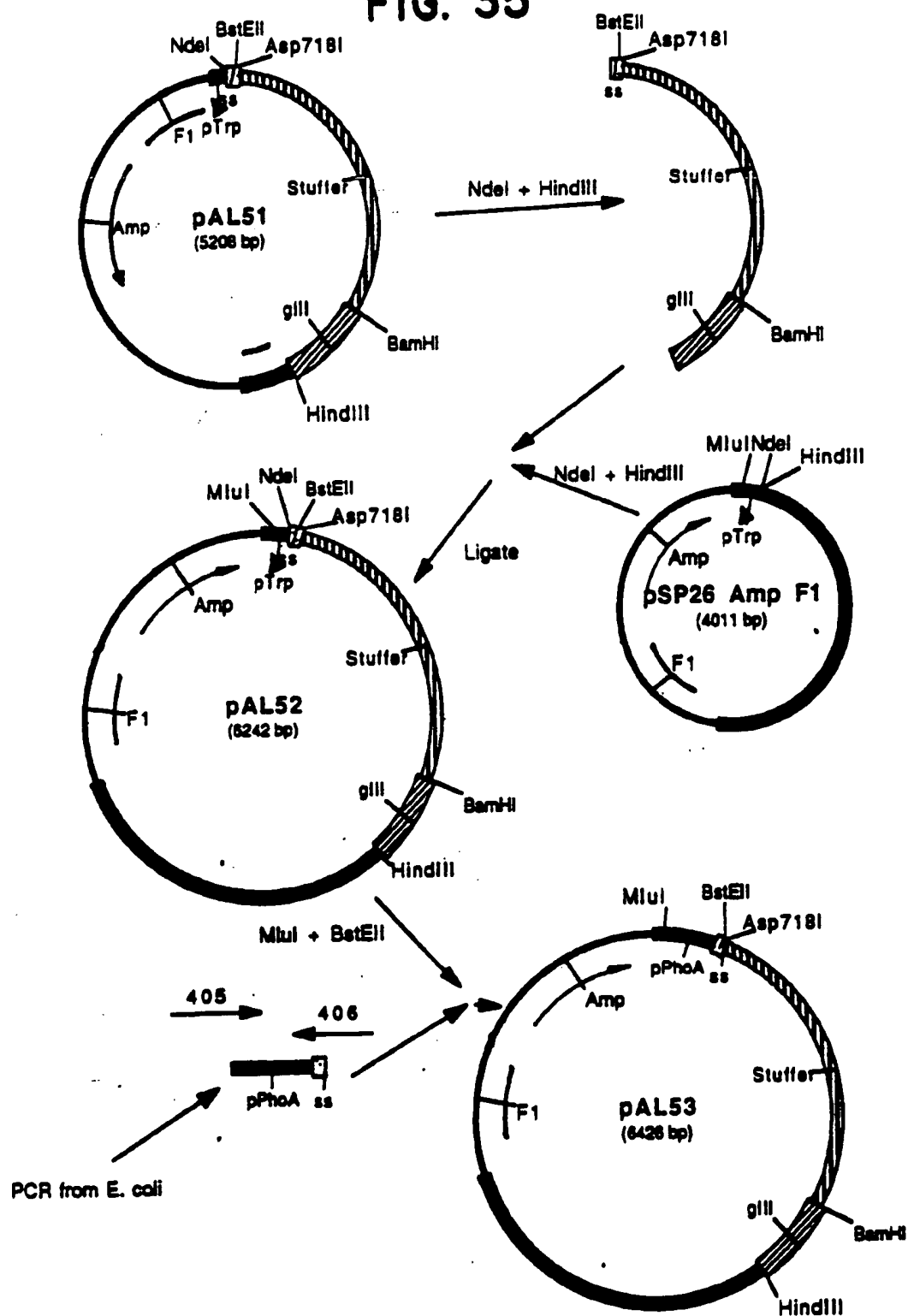


FIG. 34

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FIG. 35



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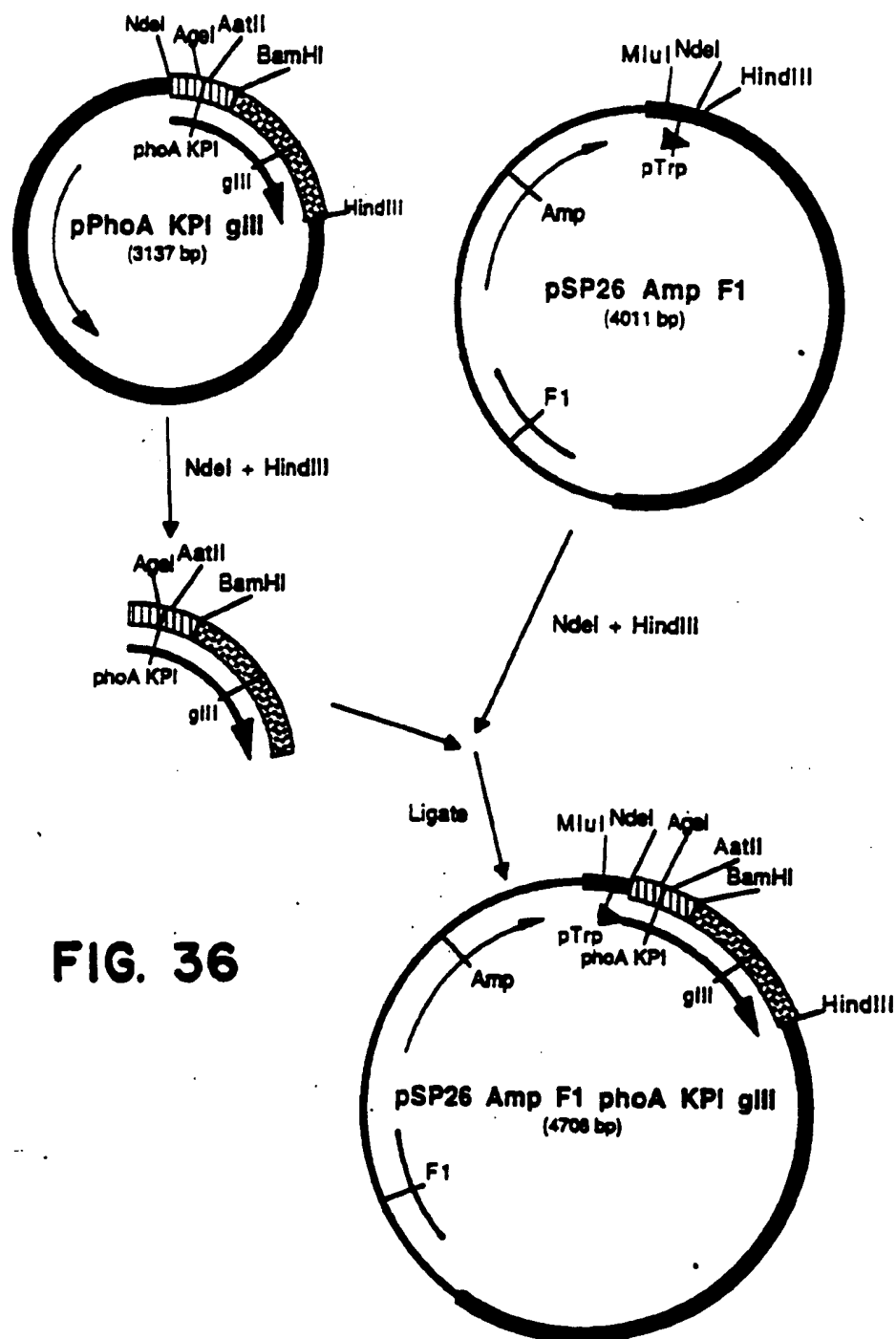
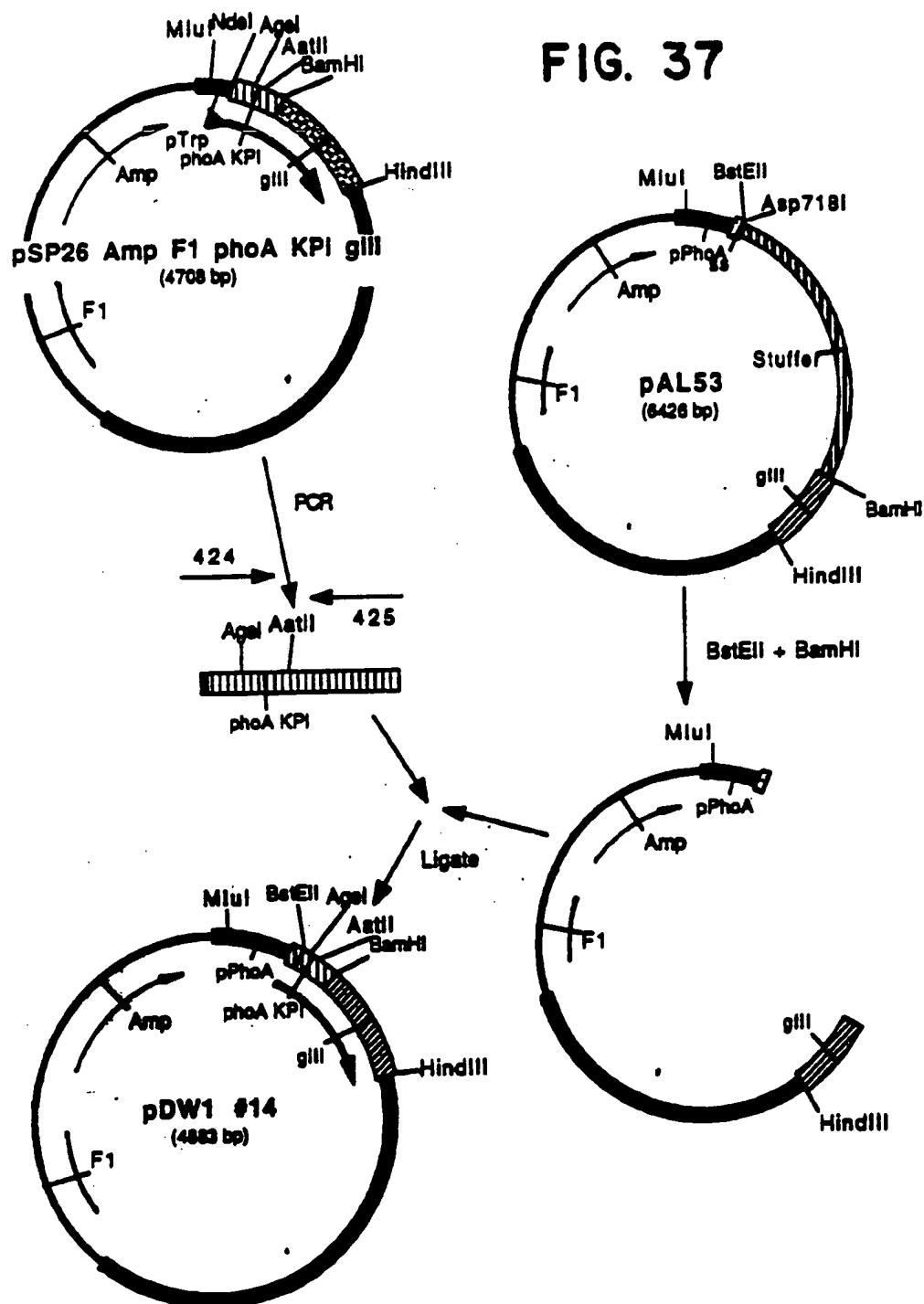


FIG. 36

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FIG. 37



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FIG. 38

phoA signal →
 GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
 Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

KPI (1-55) →
 GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CCG TGG
 Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp

AatII
 TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
 Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

BamHI →
 CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT
 Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser

qIII →
 GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA
 Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT
 Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr

GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT
 Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn

GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT
 Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
 Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA
 Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys

ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GGC ACC TTT ATG TAT GTA
 Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val

TTT TCT ACC TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
 Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser ...

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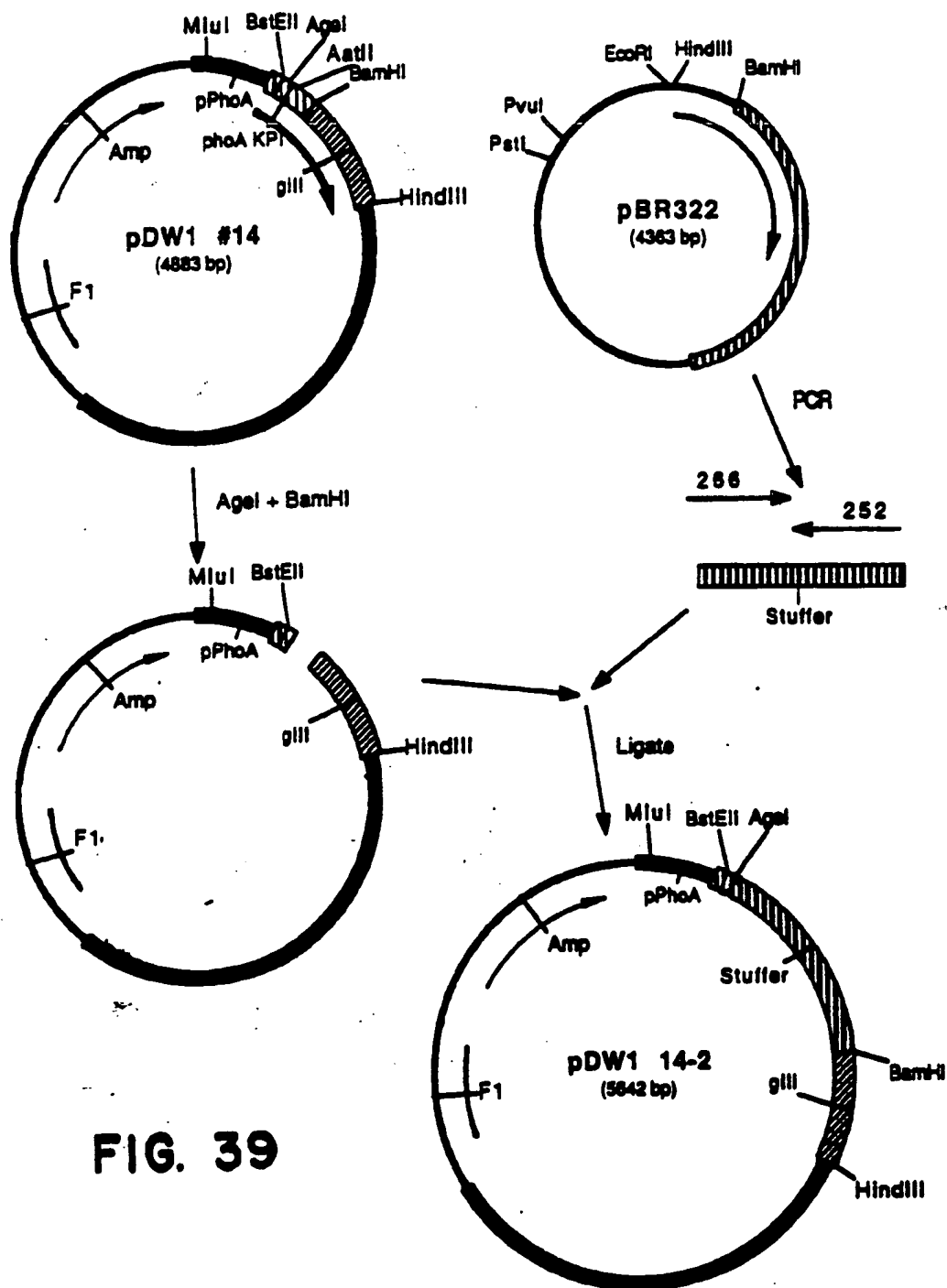
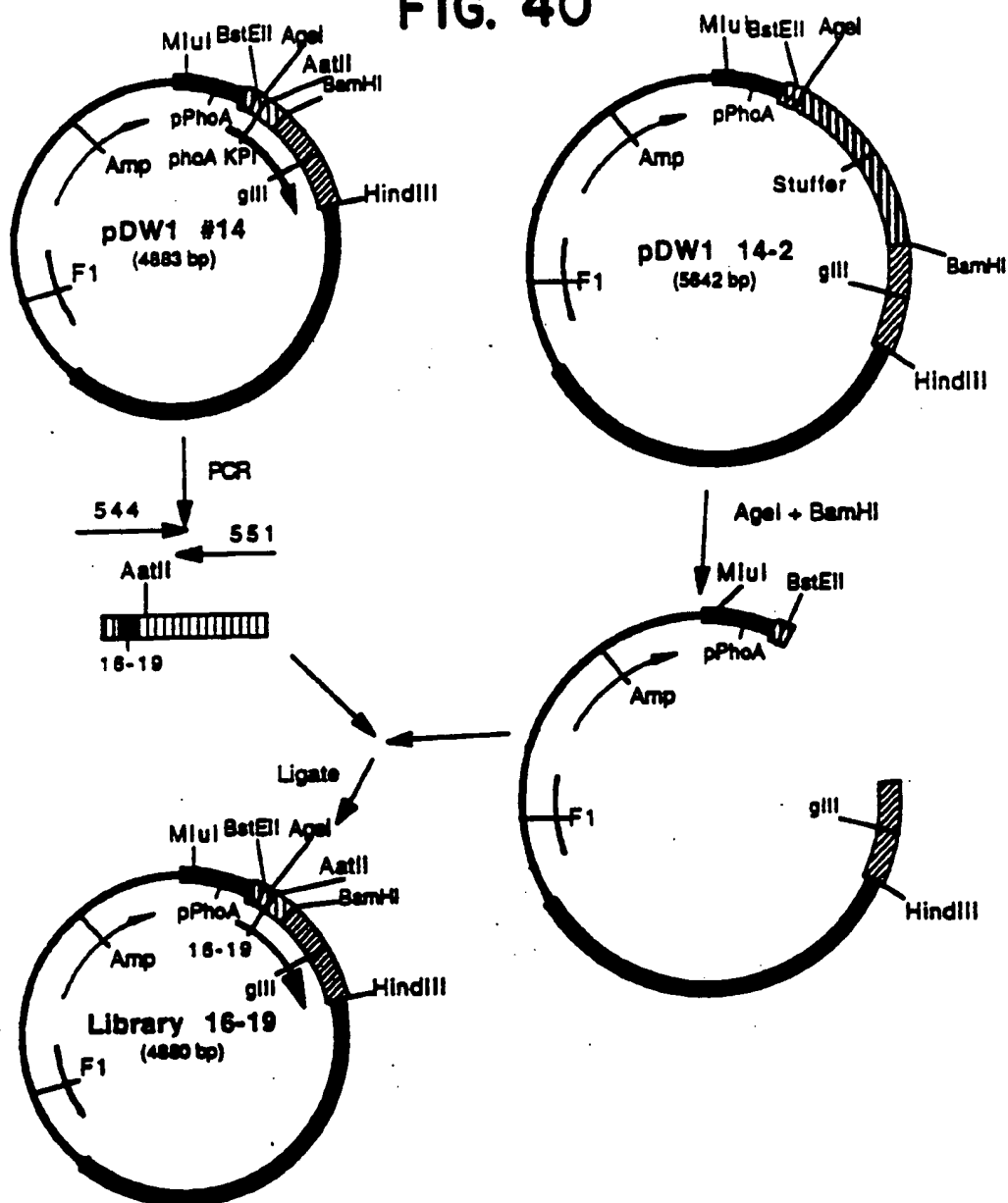


FIG. 39

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FIG. 40



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FIG. 41

phoA signal

GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
 ▶ Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

KPI (1-55; 16-19)

GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT NNS NNS NNS NNS TGG TAC
 ▶ Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg ??? ??? ??? ??? Trp Tyr

Agel

16-19

AatII

TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT
 ▶ Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg

BamHI

gIII

AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT GGT
 ▶ Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser Gly

TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT
 ▶ Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn

GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT
 ▶ Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp

TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT
 ▶ Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly

gIII

GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT
 ▶ Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn

TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT
 ▶ Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys

CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA ATA
 ▶ Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile

AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT
 ▶ Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe

TCT ACC TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
 ▶ Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser ...

FIG. 42

phoA signal →

GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
 ▶ Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

KPI (1-55; M15A, S17F)

Agel

GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TTC CGC TGG
 ▶ Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Phe Arg Trp

AatII

TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
 ▶ Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

BamHI

CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT
 ▶ Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser

gIII

GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA
 ▶ Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT
 ▶ Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr

GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT
 ▶ Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn

GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT
 ▶ Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
 ▶ Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

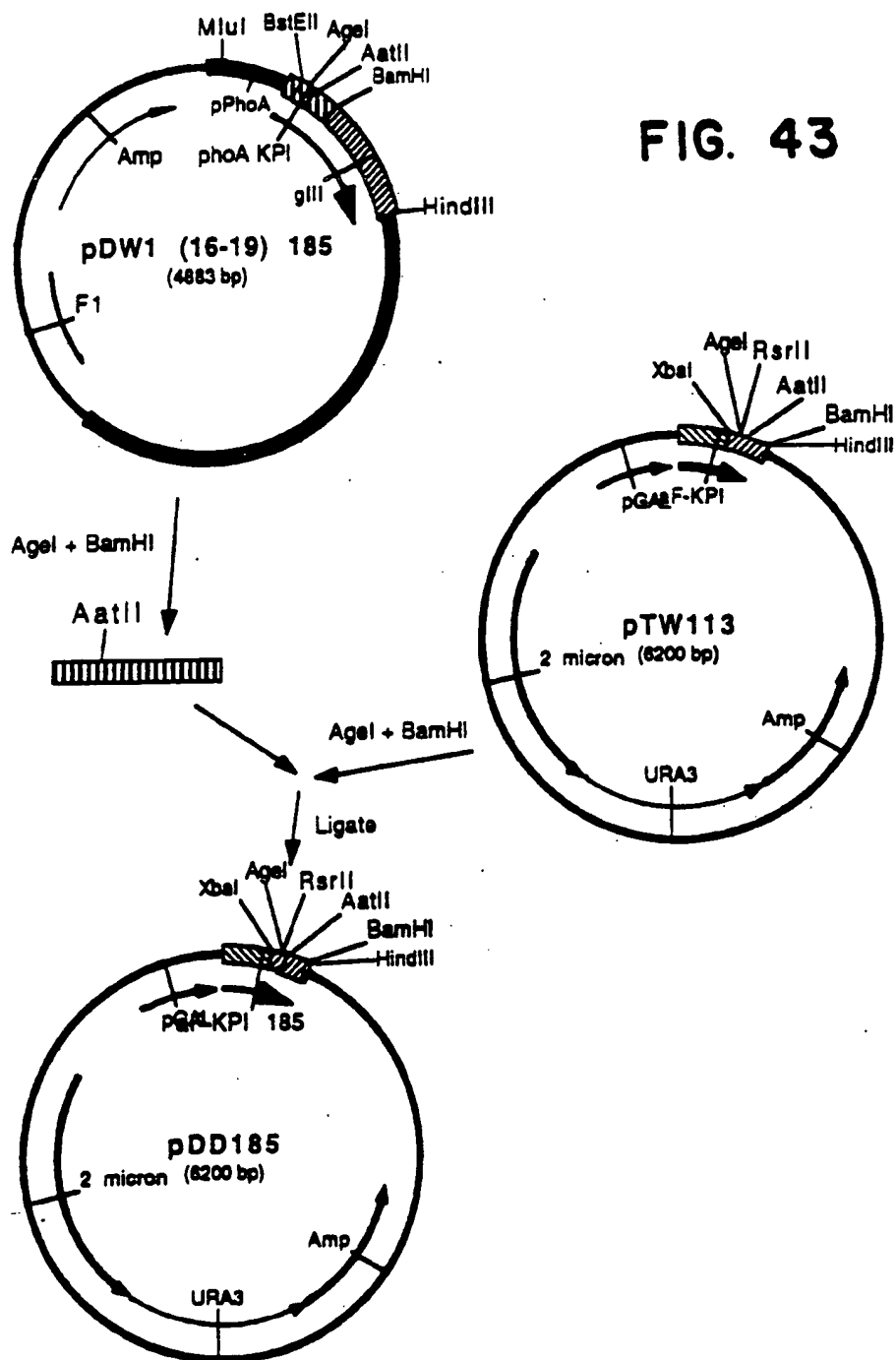
TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA
 ▶ Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys

ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA
 ▶ Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
 ▶ Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser

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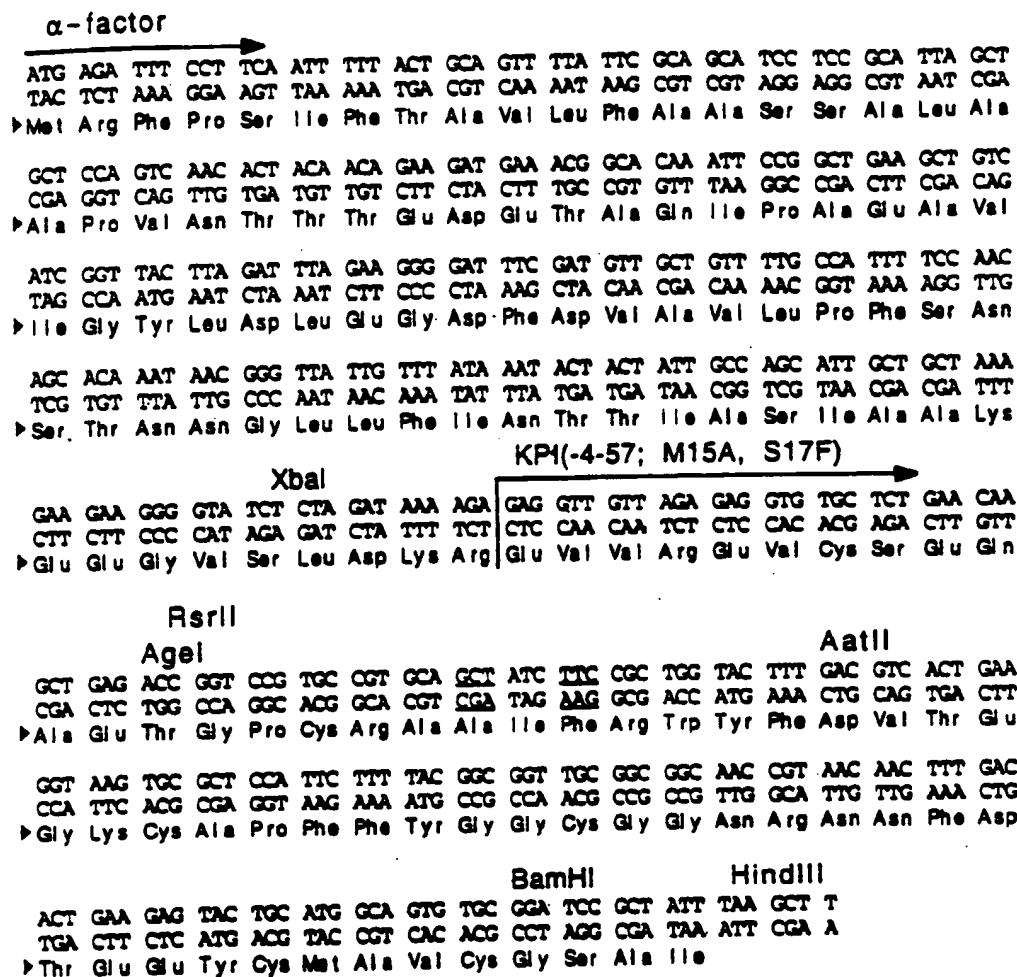
FIG. 43



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pDD185

FIG. 44



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Protease inhibition by KPI (-4-57) variants

K_{PS} (nM)

Variant	9	15	16	17	18	37	Kallikrein	X _{9a}	X _a
TW113 KPI (-4-57)							45.00	3718.0	161.0
DD185 KPI (-4-57; M15A, S17F)		A		F			0.39	150.0	196.0
TW6165 KPI (-4-57; M15A, S17W)		A		W			0.65	208.0	nd
TW6168 KPI (-4-57; M15A, S17Y)		A		Y			0.40	73.0	nd
TW6175 KPI (-4-57; M15L, S17F)		L		F			0.50	35.0	58.0
BG028 KPI (-4-57; M15L, S17Y)		L		Y			1.10	93.8	nd
TW6183 KPI (-4-57; I16H, S17F)			H	F			1.20	12440.0	159.0
TW6184 KPI (-4-57; I16H, S17Y)			H	Y			0.91	14000.0	214.0
TW6185 KPI (-4-57; I16H, S17W)			H	W			1.30	388.0	473.0
TW6173 KPI (-4-57; M15A, I16H)		A	H				1.00	1432.0	nd
TW6174 KPI (-4-57; M15L, I16H)		L	H				0.90	2796.0	nd
BG015 KPI (-4-57; M15L, S17Y, R18H)		L		Y	H		6.00	19.4	597.0
BG022 KPI (-4-57; M15A, S17Y, R18H)		L		Y	H		0.64	14.5	nd
BG029 KPI (-4-57; T9V, M15L, S17Y, R18H)	V	A		Y	H		3.20	7.9	nd
BG033 KPI (-4-57; T9V, M15A, S17Y, R18H)	V	A		Y	H		0.75	6.8	nd
DD131 KPI (-4-57; M15L, I16F, S17K)		L	F	K			7.90	1385.0	3.3
DD134 KPI (-4-57; M15L, I16F, S17K, G37Y)		L	F	K		Y	1.10	15840.0	0.6
DD135 KPI (-4-57; M15L, I16F, S17K, G37L)		L	F	K		L	1.30	7473.0	0.9

FIG. 45

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FIG. 46(1)

Inhibition KI (nM)

Variant	Sequence	P ₁ (nM)	P ₂ (nM)	P ₃ (nM)	P ₄ (nM)
Apronin	RPDCLPPPTGFCARIIIRYPYNAKAGLCQTFVYGGCRAKRNPFSAEDCHRTCGGA	20.00	0.23	5000.0	
Apronin R15, S42	DFCLPPPTGFCARIIIRYPYNAKAGLCQTFVYGGCRAKSNPFSAEDCHRTCGGA	0.91	0.17	3983.0	
KP1 (-4-57)	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	45.00	34.00	3719.0	161.0
TW6167	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	61.00		3641.0	288.0
BG031	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	34.00			
BG032	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	49.00		731.0	
TW101	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	2000.00	11.50		
TW6208	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI			368.0	
TW106	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	560.00	3.70		
DD108	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	1.70	11.20	1800.0	123.0
DD109	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	9.50		1681.0	421.0
DD110	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	2.10		624.0	55.0
DD111	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	5.80			
DD112	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	6.80		998.0	
TW6178	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	78.00		368.0	
TW6183	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	4.70	103.58	4632.0	457.0
TW6172	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	315.00			1483.0
TW6180	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	70.00		885.0	38.0
TW6181	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	150.00		1514.0	
BG001	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	38.00	10.00	489.0	204.0
TW116	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	145.00	89.00		806.0
DD102	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	16.00		315.0	
DD103	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	17.00		2128.0	110.0
DD104	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	15.00		237.0	345.0
DD105	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	18.00		198.0	320.0
TW6188	EVREVCSEQAETGFCRAIIISRMVFDVTEGKCAPFFYGGCGGNRNPFDETYCHAVCGSAI	25.80		3521.0	385.0

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Inhibition Ki (nM)

FIG. 46(2)

P. k _{eff}	Elasm ₀	X ₀	X _a
DVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	36.00		752.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	70.83		
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	54.00		277.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	110.20		89600.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI			133.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI			40.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	81.00	45.80	184.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	184.00		402.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	44.00		37.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	18.00	18.00	7972.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	216.00		1557.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	39.00		316.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	35.00		1090.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	18.00		921.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	11.00		915.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	11.00		27.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	35.00		475.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI			
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	42.00		
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	6.00	24.00	13009.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	15.00		
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	40.00		511.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	29.00		168.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	17.00		
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	7.50	18.00	1507.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	64.00		924.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	163.00		1162.0
NVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI			954.0

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FIG. 46(3)

Inhibition Ki (nM)

Variant	Sequence	P. kcal	Plasma	X ₅₀	X ₉₀
TW6139	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	19.00	22.80	152.0	78.0
TW6153	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	11.20	21.30	65.0	38.0
TW122	EVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	32.00	27.00		581.0
TW6178	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	18.00		444.0	
TW6148	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	40.00			
TW6124	EVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	64.00	48.00		
TW6149	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	54.00			
TW6173	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	1.00	7.24	1432.0	
TW6174	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.90	6.89	2796.0	
BG002	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.98	19.00	403.0	60.0
DD129	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	3.60		1884.0	6.0
DD185	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.38	8.71	150.0	198.0
TW6165	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.65	16.40	206.0	
TW6166	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.40	10.10	73.0	
BG028	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	1.10	12.10	93.8	
TW6169	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	1.20		619.0	111.0
DD113	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.85	12.80	283.0	74.0
TW6176	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.50	7.46	35.0	58.0
TW6201	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	34.60		419.0	
TW6202	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	128.60		1237.0	
TW6203	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	31.20		5045.0	
TW6204	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI			147.0	87.0
TW6205	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI			196.0	29.0
DD114	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.70	7.77	224.0	
TW6180	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	0.83	52.20	589.0	1398.0
TW6183	EVREVCSEQAETGPCRAAI SRNYFDVTEGKCA PFFYGGCGGRNNFDTSEYCHAVCGSAI	1.20	11.68	12440.0	159.0

Inhibition KI (nM)

FIG. 46(4)

Variant	Sequence	P. level	Phenol	Xia	Xa
TW6184	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	0.91	11.98	14000.0	214.0
TW6185	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	1.30	18.80	388.0	473.0
BG003	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	36.00		487.0	
TW6186	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	0.48	8.86	186.0	11.0
TW6187	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	3.80	16.40	92.0	15.0
TW6188	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	4.00		419.0	24.0
TW6189	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	4.00			34.0
TW6170	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	2.50			452.0
DD115	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI			213.0	288.0
DD170	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	0.99	18.00	550.0	
TW6176	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	3.50	118.00	56.0	
TW6177	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	7.20	32.70	245.0	156.0
BG006	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	0.30	12.10	80.0	
DD130	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	6.50			9.5
DD131	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	7.90	2.00	1385.0	3.3
DD132	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	112.00			16.8
DD120	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	8.30			11.0
DD121	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	19.00			21.0
BG014	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	9.20	18.70	18.0	
DD122	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	15.00			48.0
BG015	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	6.00	12.20	19.4	597.0
BG020	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	1.70		106.0	
BG022	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	0.84	7.26	14.5	
BG023	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	23.00		262.0	
BG024	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	4.10	7.47	38.7	
BG027	EVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGRRNPFDTSEYCHAVCGSAI	5.80		144.0	

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FIG. 46(5)

Variant	Sequence	P_{50}	Inhibition K_i (nM)	$\Delta\Delta$	$\Delta\alpha$
DD116	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	0.14	583.0		84.0
TW6191	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCYGRNNFDTSEYCHAVCGSAI	0.26	664.0		20.0
DD117	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	0.11	1034.0		98.0
BG029	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	3.20	7.9		
BG030	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	4.60	26.1		
BG033	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	0.75	5.8		
BG034	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	0.47	18.5		
BG040	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	3.40	8.6		
BG016	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	180.00	178.0		
BG017	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	180.00	200.0		
BG021	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	340.00	224.0		
BG025	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	65.00	18.2		
BG026	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	50.00	34.9		
DD118	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	0.53			
DD134	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	1.10	1.05	15840.0	0.6
DD135	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	1.30		7473.0	0.9
DD136	EVREVCSEQAETGPCRAAIFRMVFDVTEGKCAPFFYGGCGGRNNFDTSEYCHAVCGSAI	1.10			1.8

SUBSTITUTE SHEET (RULE 26)

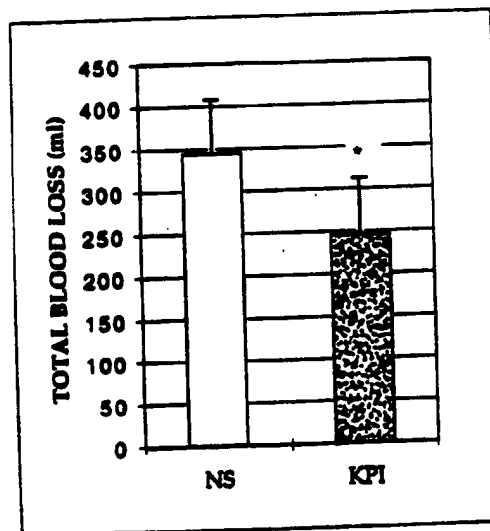
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FIG. 47

VOLUMES

NS	344.25
KPI	245.75

	KPI	NS
	298	366
	266	342
	354	294
	258	385
	168	288
	266	469
	172	338
	184	272
MEAN	245.75	344.25
STDEV	66.2414415	83.97488348
TTEST	0.009094999	



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FIG. 48

HEMOGLOBIN

NS	23.61
KPI	13.59

KPI	NS
16.58	24.95
15.19	24.87
20.21	20.46
8.99	27.59
14.63	18.23
15.31	31.59
7.7	23.26
10.14	17.96

MEAN	13.59375	23.61375
STDEV	4.261438	4.68761
TTEST		0.000536

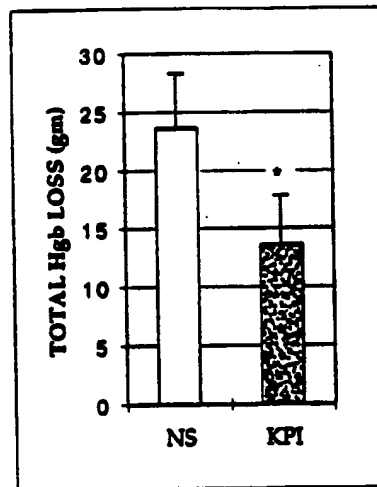


FIG. 49

PaO₂

	Baseline PaO ₂		End CPB		Obs 60 min		Obs 180 min	
	KPI	NS	KPI	NS	KPI	NS	KPI	NS
MEAN	652.2	670.9	495.7	60.5	483.7	441.3	264.1	391.3
STDEV	654	559.2	444.6	132.2	330.1	448.7	416.5	484.6
TTEST	596.2	622.9	170.2	93.8	415.4	85.1	361.9	81.3
	606.2	689.2	264.2	333.9	430.2	579.6	90.8	333.2
	633.1	665.1	567.2	341.7	613	568.3	518.2	546.6
	646.6	527	507.4	226.9	564.3	438.1	494.2	485.3
	563.2	461.7	547.1	89.1	501	42.6	452	45.6
	659.9	508	416.6	59.7	504.5	405.8	371.1	383.7
	626.425	588	426.625	167.225	480.275	369.938	150.2774	186.227
	34.46923	85.50556	140.4741	117.9931	88.61879	196.5235	p=	0.76
	p=	0.268	p=	0.0014	p=	0.17915		

N.S.

N.S.

Summary of Data

FIG. 50

Total Volumes		Serial Chest tube Hbg			
Chest tube	Sacrifice	0-30min	30-60min	60-120min	120-180min
185	113	3.7	4.3	8.6	6.2
198	68	4.3	6.4	6.7	5.7
142	212	4.1	4.4	7	7.1
190	68	2.8	4	4.4	1.9
96	72	6.3	6.5	7	6.7
188	78	4.1	6.1	5.6	6.3
134	38	3.1	4.6	5.4	4.4
158	26	6.9	5.8	5.4	4.2
MEAN		4.41	5.26	6.26	5.3
STDEV		1.45	1.04	1.32	1.72

Total volume loss		Total Hgb Loss	
KPI-1	298		16.58
KPI-2	266		15.19
KPI-3	354		20.21
KPI-4	258		8.99
KPI-5	168		14.63
KPI-6	266		15.31
KPI-7	172		7.7
KPI-8	184		10.14
MEAN		245.75	13.59
STDEV		66.24	4.26

Total Volumes		Serial Chest tube Hbg			
Chest tube	Sacrifice	0-30min	30-60min	60-120min	120-180min
274	92	7.7	8.6	6.1	5.4
236	106	7.2	7.4	7.6	7.1
252	42	5.4	7.5	7.5	6.5
303	82	8.4	7.2	7.1	6.3
140	148	7.5	7.2	5.2	5.6
261	208	4	7	7.3	7.4
218	120	7.5	7.7	5.8	4.2
206	66	7.4	8.2	6	5.3
MEAN		6.89	7.6	6.58	6.1
STDEV		1.44	1.04	0.91	0.85

Total Volumes		Total Hgb Loss	
NS-1A	366		24.95
NS-2	342		24.87
NS-3	294		20.46
NS-4	385		27.59
NS-5	288		18.23
NS-6	469		31.59
NS-7	338		23.26
NS-8	272		17.96
MEAN		344.25	23.61
STDEV		63.97	4.69

*p = 0.009 *p = 0.0005 *p = 0.004 *p = 0.0002 NS NS

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pTW 6166

FIG. 51

α -factor																							
ATG	AGA	TTT	CCT	TCA	ATT	TTT	ACT	GCA	GTT	TTA	TTC	GCA	GCA	TCC	TCC	GCA	TTA	GCT					
TAC	TCT	AAA	GGA	AGT	TAA	AAA	TGA	CGT	CAA	AAT	AAG	CGT	CGT	AGG	AGG	CGT	AAT	CGA					
Met	Arg	Phe	Pro	Ser	Ile	Phe	Thr	Ala	Val	Leu	Phe	Ala	Ala	Ser	Ser	Ala	Leu	Ala					
GCT	CCA	GTC	AAC	ACT	ACA	ACA	GAA	GAT	GAA	ACG	GCA	CAA	ATT	CCG	GCT	GAA	GCT	GTC					
CGA	GGT	CAG	TTG	TGA	TGT	TGT	CTT	CTA	CTT	TGC	CGT	GTT	TAA	GGC	CGA	CTT	CGA	CAG					
Ala	Pro	Val	Asn	Thr	Thr	Thr	Glu	Asp	Glu	Thr	Ala	Gln	Ile	Pro	Ala	Glu	Ala	Val					
ATC	GGT	TAC	TTA	GAT	TTA	GAA	GGG	GAT	TTC	GAT	GTT	GCT	GTT	TTG	CCA	TTT	TCC	AAC					
TAG	CCA	ATG	AAT	CTA	AAT	CTT	CCC	CTA	AAG	CTA	CAA	CGA	CAA	AAC	GGT	AAA	AGG	TTG					
Ile	Gly	Tyr	Leu	Asp	Leu	Glu	Gly	Asp	Phe	Asp	Val	Ala	Val	Leu	Pro	Phe	Ser	Asn					
ACC	ACA	AAT	AAC	GGG	TTA	TTG	TTT	ATA	AAT	ACT	ACT	ATT	GCC	AGC	ATT	GCT	GCT	AAA					
TGG	TGT	TTA	TTG	CCC	AAT	AAC	AAA	TAT	TTA	TGA	TGA	TAA	CGG	TCG	TAA	CGA	CGA	TTT					
Ser	Thr	Asn	Asn	Gly	Leu	Phe	Ile	Asn	Thr	Thr	Ile	Ala	Ser	Ile	Ala	Ala	Lys						
KPI(-4-57; M15A, S17Y)																							
XbaI																							
GAA	GAA	GGG	GTA	TCT	CTA	GAT	AAA	AGA	GAG	GTT	GTT	AGA	GAG	GTG	TGC	TCT	GAA	CAA					
CTT	CTT	CCC	CAT	AGA	GAT	CTA	TTT	TCT	CTC	CAA	CAA	TCT	CTC	CAC	ACG	AGA	CTT	GTT					
Glu	Glu	Gly	Val	Ser	Leu	Asp	Lys	Arg	Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln					
RsrII																							
AgeI																							
GCT	GAG	ACC	GGT	CCG	TGC	CGT	GCA	GCT	ATC	TAC	CCG	TGG	TAC	TTT	GAC	GTC	ACT	GAA					
CGA	CTC	TGG	CCA	GGC	ACG	GCA	CGT	CGA	TAG	ATG	CCG	ACC	ATG	AAA	CTG	CAG	TGA	CTT					
Ala	Glu	Thr	Gly	Pro	Cys	Arg	Ala	Ala	Ile	Tyr	Arg	Trp	Tyr	Phe	Asp	Val	Thr	Glu					
GGT	AAG	TGC	GCT	CCA	TTC	TTT	TAC	GGC	GGT	TGC	GGC	GGC	AAC	CGT	AAC	AAC	TTT	GAC					
CCA	TTC	ACG	CGA	GGT	AAG	AAA	ATG	CCG	CCA	ACG	CCG	CCG	TTG	GCA	TTG	TTG	AAA	CTG					
Gly	Lys	Cys	Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	Arg	Asn	Asn	Phe	Asp					
BamHI												HindIII											
ACT	GAA	GAG	TAC	TGC	ATG	GCA	GTG	TGC	GGA	TCC	GCT	ATT	TAA	GCT	T								
TGA	CTT	CTC	ATG	ACG	TAC	CGT	CAC	ACG	CCT	ACG	CGA	TAA	ATT	CGA	A								
Thr	Glu	Glu	Tyr	Cys	Met	Ala	Val	Cys	Gly	Ser	Ala	Ile											

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FIG. 52

α -factor																								
ATG	AGA	TTT	CCT	TCA	ATT	TTT	ACT	GCA	GTT	TTA	TTC	GCA	GCA	TCC	TCC	GCA	TTA	GCT						
TAC	TCT	AAA	GGA	AGT	TAA	AAA	TGA	CGT	CAA	AAT	AAG	CGT	CGT	AGG	AGG	CGT	AAT	CGA						
Met	Arg	Phe	Pro	Ser	Ile	Phe	Thr	Ala	Val	Leu	Phe	Ala	Ala	Ser	Ser	Ala	Leu	Ala						
GCT	CCA	GTC	AAC	ACT	ACA	ACA	GAA	GAT	GAA	ACG	GCA	CAA	ATT	CCG	GCT	GAA	GCT	GTC						
CGA	GGT	CAG	TTG	TGA	TGT	TGT	CTT	CTA	CTT	TGC	CGT	GTT	TAA	GGC	CGA	CTT	CGA	CAG						
Ala	Pro	Val	Asn	Thr	Thr	Thr	Glu	Asp	Glu	Thr	Ala	Gln	Ile	Pro	Ala	Glu	Ala	Val						
ATC	GGT	TAC	TTA	GAT	TTA	GAA	GGG	GAT	TTC	GAT	GTT	GCT	GTT	TTG	CCA	TTT	TCC	AAC						
TAG	CCA	ATG	AAT	CTA	AAT	CTT	CCC	CTA	AAG	CTA	CAA	CGA	CAA	AAC	GGT	AAA	AGG	TTG						
Ile	Gly	Tyr	Leu	Asp	Leu	Glu	Gly	Asp	Phe	Asp	Val	Ala	Val	Leu	Pro	Phe	Ser	Asn						
AGC	ACA	AAT	AAC	GGG	TTA	TTG	TTT	ATA	AAT	ACT	ACT	ATT	GCC	AGC	ATT	GCT	GCT	AAA						
TCG	TGT	TTA	TTG	CCC	AAT	AAC	AAA	TAT	TTA	TGA	TAA	CGG	TCG	TAA	CGA	CGA	TTT							
Ser	Thr	Asn	Asn	Gly	Leu	Leu	Phe	Ile	Asn	Thr	Thr	Ile	Ala	Ser	Ile	Ala	Ala	Lys						
KPI(-4-57; M15L, S17F)																								
XbaI																								
GAA	GAA	GGG	GTA	TCT	CTA	GAT	AAA	AGA	GAG	GTT	GTT	AGA	GAG	GTG	TGC	TCT	GAA	CAA						
CTT	CTT	CCC	CAT	AGA	GAT	CTA	TTT	TCT	CTC	CAA	CAA	TCT	CTC	CAC	ACG	AGA	CTT	GTT						
Glu	Glu	Gly	Val	Ser	Leu	Asp	Lys	Arg	Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln						
RsrII																								
AgeI																								
GCT	GAG	ACC	GGT	CCG	TGC	CGT	GCA	TTG	ATC	TTT	CGC	TGG	TAC	TTT	GAC	GTC	ACT	GAA						
CGA	CTC	TGG	CCA	GGC	ACG	GCA	CGT	AAC	TAG	AAG	GCG	ACC	ATG	AAA	CTG	CAG	TGA	CTT						
Ala	Glu	Thr	Gly	Pro	Cys	Arg	Ala	Leu	Ile	Phe	Arg	Trp	Tyr	Phe	Asp	Val	Thr	Glu						
GCT	AAG	TGC	GCT	CCA	TTC	TTT	TAC	GGC	GGT	TGC	GGC	GGC	AAC	CGT	AAC	AAC	TTT	GAC						
CCA	TTC	ACG	CGA	GGT	AAG	AAA	ATG	CCG	CCA	ACG	CCG	CCG	TTG	GCA	TTG	TTG	AAA	CTG						
Gly	Lys	Cys	Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	Arg	Asn	Asn	Phe	Asp						
BamHI																								
ACT	GAA	GAG	TAC	TGC	ATG	GCA	GTG	TGC	GGA	TGC	GCT	ATT	TAA	GCT	T									
TGA	CTT	CTC	ATG	ACG	TAC	CGT	CAC	ACG	CCT	AGG	CGA	TAA	ATT	CGA	A									
Thr	Glu	Glu	Tyr	Cys	Met	Ala	Val	Cys	Gly	Ser	Ala	Ile												
HindIII																								

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FIG. 53

α -factor																			
ATG	AGA	TTT	CCT	TCA	ATT	TTT	ACT	GCA	GTT	TTA	TTC	GCA	GCA	TCC	TCC	GCA	TTA	GCT	
TAC	TCT	AAA	GGA	AGT	TAA	AAA	TGA	CGT	CAA	AAT	AAG	CGT	CGT	AGG	AGG	CGT	AAT	CGA	
Met	Arg	Phe	Pro	Ser	Ile	Phe	Thr	Ala	Val	Leu	Phe	Ala	Ala	Ser	Ser	Ala	Leu	Ala	
GCT	CCA	GTC	AAC	ACT	ACA	ACA	GAA	GAT	GAA	ACG	GCA	CAA	ATT	CCG	GCT	GAA	GCT	GTC	
CGA	GGT	CAG	TTG	TGA	TGT	TGT	CTT	CTA	CTT	TGC	CGT	GTT	TAA	GGC	CGA	CTT	CGA	CAG	
Ala	Pro	Val	Asn	Thr	Thr	Thr	Glu	Asp	Glu	Thr	Ala	Gln	Ile	Pro	Ala	Glu	Ala	Val	
ATC	GGT	TAC	TTA	GAT	TTA	GAA	GGG	GAT	TTC	GAT	GTT	GCT	GTT	TTG	CCA	TTT	TCC	AAC	
TAG	CCA	ATG	AAT	CTA	AAT	CTT	CCC	CTA	AAG	CTA	CAA	CGA	CAA	AAC	GGT	AAA	AGG	TTG	
Ile	Gly	Tyr	Leu	Asp	Leu	Glu	Gly	Asp	Phe	Asp	Val	Ala	Val	Leu	Pro	Phe	Ser	Asn	
AGC	ACA	AAT	AAC	GGG	TTA	TTG	TTT	ATA	AAT	ACT	ACT	ATT	GCC	AGC	ATT	GCT	GCT	AAA	
TGG	TGT	TTA	TTG	CCC	AAT	AAC	AAA	TAT	TTA	TGA	TGA	TAA	CGG	TGG	TAA	CGA	CGA	TTT	
Ser	Thr	Asn	Asn	Gly	Leu	Leu	Phe	Ile	Asn	Thr	Thr	Ile	Ala	Ser	Ile	Ala	Ala	Lys	
XbaI										KPI(-4-57; M15L, S17Y)									
GAA	GAA	GGG	GTA	TCT	CTA	GAT	AAA	AGA	GAG	GTT	GTT	AGA	GAG	GTG	TGC	TCT	GAA	CAA	
CTT	CTT	CCC	CAT	AGA	GAT	CTA	TTT	TCT	CTC	CAA	CAA	TCT	CTC	CAC	ACG	AGA	CTT	GTT	
Glu	Glu	Gly	Val	Ser	Leu	Asp	Lys	Arg	Glu	Val	Val	Arg	Glu	Val	Cys	Ser	Glu	Gln	
RsrII										AatII									
AgeI																			
GCT	GAG	ACC	GGT	CCG	TGC	CGT	GCA	TTG	ATC	TAC	CCG	TGG	TAC	TTT	GAC	GTC	ACT	GAA	
CGA	CTC	TGG	CCA	GGC	ACG	GCA	CGT	AAC	TAG	ATG	CCG	ACC	ATG	AAA	CTG	CAG	TGA	CTT	
Ala	Glu	Thr	Gly	Pro	Cys	Arg	Ala	Leu	Ile	Tyr	Arg	Tyr	Tyr	Phe	Asp	Val	Thr	Glu	
GCT	AAG	TGC	GCT	CCA	TTT	TAC	GGC	GGT	TGC	GGC	GGC	AAC	CGT	AAC	AAC	TTT	GAC		
CCA	TTC	ACG	CGA	GGT	AAG	AAA	ATG	CCG	CCA	ACG	CCG	CCG	TTG	GCA	TTG	TTG	AAA	CTG	
Gly	Lys	Cys	Ala	Pro	Phe	Phe	Tyr	Gly	Gly	Cys	Gly	Gly	Asn	Arg	Asn	Asn	Phe	Asp	
BamHI										HindIII									
ACT	GAA	GAG	TAC	TGC	ATG	GCA	GTG	TGC	GGA	TCC	GCT	ATT	TAA	GCT	T				
TGA	CTT	CTC	ATG	ACG	TAC	CGT	CAC	ACG	CCT	AGG	CGA	TAA	ATT	CGA	A				
Thr	Glu	Glu	Tyr	Cys	Met	Ala	Val	Cys	Gly	Ser	Ala	Ile							

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FIG. 54(1)

PROTEIN	SEQUENCES	K _i kallikrein	K _i Factor XIIa	K _i Plasmin
Aprotinin	RFDFCLEPPTTQPCRAHIIIRYFYNKAGLCQTFVYGGCRKRNHFKSAZDCHRTGGGA	22.6	5000	0.33
KPI (-4-57)	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	45.0	3710.0	34.00
TM101	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	>5000	nd	12.30
TM106	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	449.0	nd	2.98
TM116	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	116.00	nd	70.90
TM105	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	>5000	nd	1.45
TM117	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	>5000	nd	19.90
TM115	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	671.0	nd	2.24
TM102	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	>5000	nd	1.27
CL005	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	>5000	>5000	>5000
TM6172	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	315.0	nd	1555.0
TM6207	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	54.0	635.0	44.10
CL0062	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	110.2	89600	31.10
DD108	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	1.7	1600.0	11.20
DD110	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	2.1	624.0	11.000
DD111	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	5.6	nd	nd
DD112	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	6.8	998.0	nd
DD102	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	16.0	315.0	nd
DD103	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	17.0	2128.0	nd
DD104	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	15.0	237.0	nd
DD105	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	18.0	198.0	nd
TM6166	EVVREVCSEQAETGPCRAHIIIRNYFDVTEGKCAPFFYGGCGGNHNFDTSEYCHAVCGSAI	0.4	73.0	10.10

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FIG. 54(2)

TM6165	EVVREVCSEQAEIUGCRAAIIHNTIFDVTGKCAFFYGGCGGNNHFDTEIYCHAVCGSAI	.65	206.0	16.4
BQ28	EVVREVCSEQAEIUGCRAAIIHNTIFDVTGKCAFFYGGCGGNNHFDTEIYCHAVCGSAI	1.1	93.8	12.10
TM6175	EVVREVCSEQAEIUGCRAAIIHNTIFDVTGKCAFFYGGCGGNNHFDTEIYCHAVCGSAI	0.5	35.0	7.46
TM6238	EVVREVCSEQAEIUGCRAAIIHNTIFDVTGKCAFFYGGCGGNNHFDTEIYCHAVCGSAI	2.5	40.0	nd
TM6245	EVVREVCSEQAEIUGCRAAIIHNTIFDVTGKCAFFYGGCGGNNHFDTEIYCHAVCGSAI	9.9	76	nd
TM6247	EVVREVCSEQAEIUGCRAAIIHNTIFDVTGKCAFFYGGCGGNNHFDTEIYCHAVCGSAI	4.6	30	nd

